THE UNIVERSITY OF ALBERTA

MUTAGENIC ACTION OF TWO INDAZOLE ANALOGS ON *SACCHAROMYCES CEREVISIAE*

by

© MAVIS GAIL MEADOWS

A THESIS

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ABSTRACT

Two indazole analogs, hycanthone and IA-4, both effective drugs against the disease schistosomiasis, have been tested for mutagenicity in the yeast *Saccharomyces cerevisiae*. Hycanthone has been shown to induce reversions in the haploid strain, XV169-15A, for the mutant alleles, *his I-7*, *hom 3-I0*, and *lys I-I*, which are a presumptive missense mutation, frameshift mutation, and nonsense mutation, respectively. This mutagenic effect was observed whether time of exposure to the drug was the variable or the concentration of the drug was the variable.

IA-4 was not found to be mutagenic in this system under the experimental conditions used, but was seen to be toxic to the yeast.

Hycanthone was also shown to induce intragenic recombination and mitotic crossing-over in the diploid strain of yeast, X841. The mutagenicity and toxicity of hycanthone were enhanced by higher pH and by light.

From this study, it is concluded that hycanthone is a general mutagen for *Saccharomyces cerevisiae*.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table | Page

LIST OF FIGURES

Figure Page

Figure Page

INTRODUCTION

Two compounds of related structure, which have been sent to our laboratory, have been tested for mutagenicity in the yeast *Saccharomyces cerevisiae*. One of the chemicals, hycanthone, also called IA-2, has already been administered to more than 300,000 people in Africa and South America as a specific chemotherapeutic agent against the disease schistosomiasis. The other compound, called IA-4, has not as yet been used against the disease in other than laboratory animals.

These drugs are only two out of approximately 250,000 which have already been used against the disease, most of them being metallic derivatives. But the need for chemotherapy is great as the disease incidence is now on the increase in the African and South American nations. The ideal drug to be used against the disease would pose no toxic, mutagenic, carcinogenic, or teratogenic threats to the infected people (Eislager, 1970).

The Disease: Schistosomiasis

Schistosomiasis, also called bilharziasis, or snail fever, is a disease caused by infection from parasitic schistosomatoid trematodes. These flatworms, which use snails as intermediate hosts and man and higher animals as final hosts, can have life spans as long as thirty years, and cause considerable damage to their hosts during that time. There are three species that infect humans: *Schistosoma mansoni*, *Schistosoma japonicum*, and *Schistosoma hematobium*.

Schistosoma mansoni is common in Africa, South America, and the West Indies. *Schistosoma japonicum* is prevalent in Asia, and *Schistosoma*

hematobium is common in Africa and in interior Asia. Cases have also been reported in New York, Chicago, Philadelphia, and other large cities among immigrants from tropical areas.

The infection is started by entry of cercariae, which are free-swimming larvae, through the host's skin, if contacted externally, or through the lining of the throat and mouth if swallowed in drinking water. From the point of penetration they enter the bloodstream, travel to the lungs by way of the heart, sojourn for a time in the liver, and then move to the mesenteric veins for breeding.

The manifestations of the disease vary from local tissue reactions to death. The disease usually does not kill quickly, rather, it causes chronic ill health over a number of years in its victims.

The migration of eggs from the worms through the tissue walls, to be released in the feces, leads to inflammations, necroses, and abscesses if the eggs become lodged in the mucosa. Hemorrhage and diarrhea are common results of egg penetration into the colon. In geographical areas where reinfection is common, repeated penetrations by the eggs leads to such extensive scar formation in the intestines and the bladder that the eggs must be transported to other organs. Wherever these eggs become lodged, there is inflammation and fibrosis.

In cases of heavy infection, mature worms migrate to organs other than the colon, for example to the brain, oviducts, lungs, and gonads, causing pathological changes. Such severe cases usually cause death.

With malaria and hookworm diseases being brought under control by insecticides, drugs, and public health measures, the World Health Organization has turned its attention to the increasing number of cases of

schistosomiasis. Its aim is to control the disease through snail eradication, environmental sanitation, and chemotherapy. The first real opportunity to practice modern experimental chemotherapy on the disease came in 1932 when researchers at the Bayer Elberfeld Laboratories perfected a technique for infecting small laboratory animals with *S. mansoni* which in turn could be used for screening a large number of drugs.

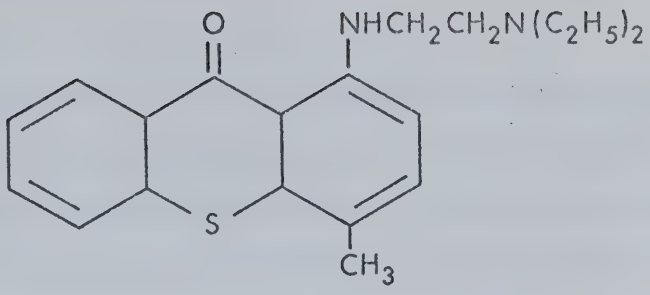
Lucanthone

A number of related compounds were synthesized by the Bayer Elberfeld Laboratories and these were called Miracil A, B, C, and D. Of these, Miracil D appeared to be the most effective drug against schistosomiasis. Miracil D, also called lucanthone, is 1-β-diethylamino-4-methyl-thioxanthone hydrochloride. It is an odorless, bright yellow compound and has the structure shown in Figure 1. It was the first nonmetallic compound to be used against the disease (Blair, 1958).

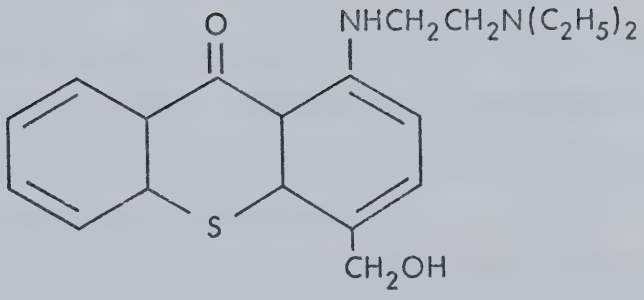
Despite its antischistosomal activity, lucanthone has strong side effects. In 1945, work done by Kigurth and Gonnet demonstrated that rabbits, which tolerated the drug well, had signs of degeneration of organs, especially the heart and kidneys. In cats, this effect was even more pronounced. Other laboratory animals proved to be intolerant to the drug because its use resulted in diarrhea and vomiting. Because of the harshness of schistosomiasis, test cases were done on infected people in some countries. In humans, the effectiveness of lucanthone varied; those who were able to complete a course of treatment were usually cured, but most victims were not able to complete the course of treatment because of the severity of the side effects, particularly those of a

Figure 1. Structures of lucanthone, hycanthone, and IA-4.

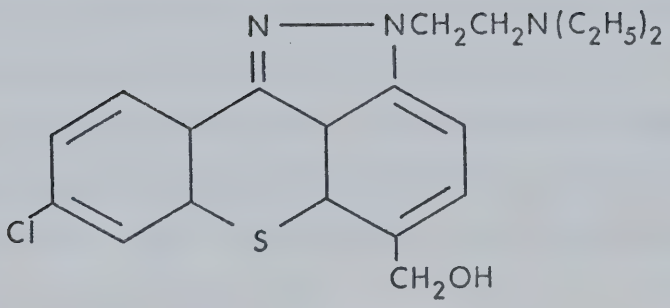
Lucanthone
IA - 1



Hycanthone
IA - 2



IA - 4



psychoneurologic nature.

Besides the gross effects on the physiology of individuals, lucanthone has been found to have some interesting effects at the molecular level. Weinstein (1965), using *Bacillus subtilis*, showed that 20 micrograms per milliliter of lucanthone, by virtue of its binding with nucleic acids, blocked DNA-dependent RNA synthesis and RNA-dependent protein synthesis. Weinstein (1967) also showed that it was active in *Escherichia coli* as an inhibitor of growth and of RNA synthesis, and that it partially inhibited DNA and protein synthesis. The action of lucanthone was comparable to that of actinomycin D in that it inhibited growth by blocking DNA-directed RNA synthesis. Weinstein (1967) also showed that spermine, which antagonizes the action of proflavine and quinacrines, also interfered with the action of lucanthone.

Bases (1969), using HeLa-S-3 cells, found that after a two-hour treatment with lucanthone at three milligrams per milliliter, RNA synthesis was depressed to 21 per cent and DNA synthesis to 27 per cent of their former levels, but protein synthesis was not inhibited. Although lucanthone was not as specific as actinomycin D, it was more advantageous in his experiments in that the action of lucanthone was reversible, and it was 5000 times less potent in killing the cells. Bases (1970) also found that the HeLa cells which had been irradiated with 300 or 500 rad of X-rays lost viability more rapidly after exposure to the drug; apparently, lucanthone inhibits post-irradiation repair.

Lüers (1955) had earlier shown that lucanthone causes chromosome breakage and induces recessive lethal mutations in Drosophila. U (1972) has also shown that lucanthone can induce loss of chromosomes in Drosophila

males both by itself and in conjunction with radiation.

Because of the undesirable clinical side-effects, and because of the molecular lesions it induces, lucanthone came to be regarded as being of limited value for treatment of schistosomiasis. Also it was suspected that the chemotherapeutic effects of the drug were mediated through a therapeutically more active metabolite. It was found that the drug underwent extensive metabolic transformations in all species in which it was used. That is, the principal products found in the urine differed from species to species. In the mouse, it is a sulfone derivative, whereas in the monkey it is a sulfoxide. In man, it becomes a chemopeptide (a sulfoxide plus a polypeptide), but none of these qualified as the active metabolite (Berberian *et al.*, 1967).

Aspergillus sclerotorium converts lucanthone into a mixture of new substances whose structures were established by chemical and spectroscopic methods. The major product was a hydroxymethyl compound which had the characteristic of being extremely sensitive to acid (Rossi *et al.*, 1965, 1967). This compound was named hycanthone, which is 1-{[2-(Diethylamino) ethyl] amino}-4-(hydroxymethyl)-thioxanthen-9-one monomethane sulfonate.

When the urinary products of mice and monkeys, which had been given lucanthone, were again examined, this time without acid treatment, hycanthone was indeed found to be the major breakdown product of lucanthone. Furthermore, it was shown to be biologically more active than lucanthone (Rossi *et al.*, 1967). That is, hycanthone is three times more active than lucanthone against schistosomiasis in mice and eight times more active against schistosomiasis in hamsters.

Hycanthone

Preliminary tests were performed in Africa and Brazil with hycanthone

on victims of schistosomiasis (Katz *et al.*, 1968). Individuals were given tablets orally and it was found that there was a satisfactory regression of clinical symptoms relevant to schistosome infection. It took three or four weeks before 80 per cent of the patients were cured. The only side effects were those associated with gastrointestinal disorders, i.e., nausea, vomiting, anorexia, and headaches. The dangerous psychoneurological side-effects noticed after lucanthone treatment were not seen after administration of hycanthone. Intramuscular injections were also initiated and it was found that a single injection gave more than 90 per cent cure without appreciable side-effects. Compared to lucanthone, there is a good clinical tolerance for hycanthone.

1. *Physiological action of hycanthone.* Hernandez *et al.* (1971), using tritiated hycanthone, reported that the patterns of absorption and distribution of hycanthone in tissues and organs is similar in rats and monkeys, the peak being at 30 minutes after intramuscular injection. There is almost complete absorption away from the site of injection by 72 hours. Thin layer chromatography and scanning of radioactive material showed that unchanged hycanthone is usually found in tissues and blood.

After injection of the drug, the flatworms move from the mesenteric vein to the liver within 24 hours (Yarinsky *et al.*, 1970). The worms appear to be able to concentrate large amounts of the drug, so it has been concluded that the toxicity of hycanthone for adult schistosomes may be attributable to the action of the unchanged drug, and not of a metabolized product of hycanthone. Olivier (1969) reported that the worms appear to accumulate hycanthone in the gut; he reported that a single intramuscular injection of hycanthone is as effective as a five-day oral regimen of

hycanthone.

Recently a multidisciplinary group of people met, under the auspices of the World Health Organization, to review the use of the drug hycanthone and they concluded that, since hycanthone was expected to be used on children and young adults, it should be tested for its potential mutagenic activity. This group also concluded that many other drugs which appear to be effective against schistosomiasis should also be tested for mutagenicity. One of these is IA-4: 8-chloro-2-[2-(diethylamino)ethyl]-2H[1]benzothiopyrano[4,3,2-cd]-indazole-5-methanol monomethananesulfonate.

2. *Mutagenicity of hycanthone.* In order to know whether hycanthone was mutagenic in mammalian cells, Clive *et al.* (1972) used a genetic system of L5178 Y mouse lymphoma cells, and looked for forward mutations at the thymidine kinase (TK) locus in heterozygous mutant cells, TK/tk, as a function of hycanthone concentration. This group carried out two experiments on two independent cultures; the results showed that hycanthone-treated cultures had increased frequencies of mutant cells which were induced approximately proportionally to the concentration of hycanthone. Further, the elevation of mutant frequency induced by 10^{-4} M hycanthone was equal to approximately 10^{-3} M ethyl methanesulfonate or 120 rads of X-rays administered under identical conditions.

Hartman *et al.* (1971) chose 13 mutants of the histidine operon in *Salmonella typhimurium* to use in a spot test which was employed to screen for mutagenicity of hycanthone and lucanthone. They tested two salts of hycanthone, the monomethanesulfonate salt and the furoate, as well as lucanthone. The two hycanthone compounds were found to behave identically

in their system. In tests with five representative frameshift mutants, hycanthone was effective in inducing reversions; lucanthone was not. These five mutants also reverted after exposure to ICR-191 and three of them reverted after exposure to nitrosoguanidine. Any other strains that had failed to revert after exposure to ICR-191 also did not revert after hycanthone treatment.

In the phage T4, growing in strain K12 of *Escherichia coli*, hycanthone increased the reversion frequency for two ICR-191-revertible frameshift mutations in the rII region. Lucanthone was also found to be a mutagen in this test system.

Hartman *et al.* (1973), using their Salmonella and T4 phage systems again, found that the number of mutations induced by hycanthone is proportional to dose over a 250-fold range. As a minimal estimation, they stated that one mutation is induced for each microgram of hycanthone added to an agar plate, and that only two hours of exposure to the drug is necessary to induce and fix the reversion. Upon retesting the two compounds with 28 other spontaneous, revertible frameshift strains, or missense strains of Salmonella, they did not find lucanthone to be mutagenic. They verified that hycanthone would induce frameshift mutations only. However, both compounds inhibit the growth of Salmonella and to the same extent. Again, hycanthone and lucanthone are both mutagenic in T4.

Hartman *et al.* also found that IA-4 is approximately ten per cent as mutagenic as hycanthone for a very sensitive frameshift mutant.

Brusick and Zeiger (1972) used a plate-test method, which involved placing a mutagen-saturated filter-paper disk on agar plates previously plated with mutant strains of microorganisms. He observed the revertants

appearing around the disks, and compared the procaryote, *Salmonella typhimurium*, and the eucaryote, *Saccharomyces cerevisiae*, in their reversion patterns of the mutants. Among other compounds, hycanthone was tested with this system. His experiments indicated that hycanthone was not mutagenic in yeast. Under the conditions of his experiments, hycanthone induced neither base substitution mutations nor frameshift mutations, but it was mutagenic in Salmonella for frameshift mutations only.

Purpose of Study

Since Brusick's plate-test method is qualitative only, as he admits himself, it is obvious that a more quantitative system should be used to test the mutagenicity of hycanthone in yeast. Further, Dr. Ernest Bueding and Dr. F.J. de Serres (personal communications) suggested that pH may be a critical factor in hycanthone mutagenesis. Since pH was not stringently controlled in the plate-test method of Brusick, the mutagenic action of hycanthone was tested in yeast at pH 7.0 and was found to be mutagenic on all markers tested. Hycanthone was also tested at pH 5.9 so that it could be compared to the action of 1A-4, which does not dissolve above pH 6.0. Also an experiment was done to establish if there is a differential mutagenic activity of hycanthone under different conditions of light.

MATERIALS AND METHODS

A. Materials

1. Strains of Yeast

The two following strains of the yeast *Saccharomyces cerevisiae* were selected for the mutagenesis testing.

(a) XV169-15A is a haploid strain of yeast constructed by R.C. von Borstel, which has the following genotype:

a trp5-48 arg4-17 lys1-1 ade2-1 hom3-10 his1-7.

Four of the markers (*trp5-48*, *arg4-17*, *lys1-1*, and *ade2-1*) are suppressible mutants of the ochre variety (Hawthorne, 1969). Reversions of these individually are a result of a base substitution at the locus itself; reversions in two or more of these at one time are the result of a change in a tRNA molecule, which causes a "super-suppression." The change in the tRNA molecule can be either due to a base substitution or an addition or deletion of a base (Magni *et al.*, 1966).

The mutant *his1-7* was isolated by Hurst and Fogel (1964). It is osmotically remedial (Hawthorne and Friis, 1964), does not have an enhanced reversion rate during meiosis (Fogel and Hurst, unpublished data), and its revertant colonies are heterogenous: i.e. their growth rates vary, and some of the revertants are often associated with feeder colonies. For these reasons *his1-7* is believed to be a missense mutant.

The *hom3-10* mutant was isolated by Magni (1969). Because its reversion rate during meiosis is 27.5 times higher than during mitosis, Magni believes it to be a frameshift mutation.

(b) X841 is a diploid strain, obtained from R.K. Mortimer, and was developed as a hybrid between two related haploid strains. Its genotype is:

$$\frac{a}{\alpha}\ \frac{his5\text{-}2}{his5\text{-}2}\ \frac{trp1\text{-}1}{trp1\text{-}1}\ \frac{+}{ade2}\ \frac{+}{pet1}\ \frac{arg4\text{-}1}{+}\ \frac{+}{arg4\text{-}2}\ \frac{thr}{+}\ \frac{met}{+}\ \frac{+}{ura}\ \frac{lys}{+}\ \frac{+}{leu1.}$$

α and *a* represent complementary mating type alleles. The symbols *his*, *trp*, *ade*, *arg*, *thr*, *met*, *ura*, *lys*, and *leu* indicate the inability to grow in the absence of histidine, tryptophane, adenine, arginine, threonine, methionine, uracil, lysine, and leucine, respectively. Of principal interest for our study was the *arg4* locus, because it is allelic for *arg4-1* and *arg4-2* and its reversion frequency would be a measure of intragenic recombination. Among colonies arising on complete medium from the treated X841 cells are some showing segregation for the *ade2* locus. This marker, when homozygous or hemizygous, results in formation of red pigment. Sectored red/white and whole red colonies arise mostly from induced mitotic crossing-over between *ade2* and the centromere. A small proportion of the variant colonies would also result from gene conversion or haploidization.

2. Media

YEPD: a complex medium used for routine growth of cultures and for viability assays; 1% Difco yeast Extract, 2% Bacto-peptone, 2% dextrose, 2% Bacto-agar.

Minimal Medium plus Vitamins (MV): 0.67% yeast nitrogen base without amino acids; 2% glucose, 2% sugar.

Mortimer Complete Medium (MC): the minimal medium plus vitamins supplemented with the following amino acids in the given quantities: adenine, arginine, lysine, histidine, tyrosine, methionine, tryptophan, uracil, all at a concentration of 20 mg/litre; serine at 375 mg/litre; leucine at 30 mg/litre; and threonine at 350 mg/litre (von Borstel *et al.*, 1971).

Reversion from prototrophy to auxotrophy was measured by using omission medium, which is MC lacking one of the supplements; so that the

notations -lys, -his, -thr, -arg represent MC lacking lysine, MC lacking histidine, MC lacking threonine, and MC lacking arginine, respectively.

3. Chemicals

The hycanthone monomethanesulfonate was a gift from Dr. S. Archer of the Winthrop-Sterling Pharmaceutical Company. Some hycanthone had previously been supplied by Dr. E. Bueding of Johns Hopkins University, when he had also sent a small sample of IA-4. Further amounts of IA-4 were supplied by Dr. E.F. Elslager of Parke, Davis and Company, Ann Arbor, Michigan.

Hycanthone was found to readily dissolve at all pH's lower than pH8 where it began to precipitate. IA-4 precipitated at pH6.1 and therefore was used at a pH5.9.

Generally the hycanthone solutions were made up at a concentration of 1 mg/ml, for all dose-action studies which were done using time of exposure to the drug as the variable.

When the concentration of the hycanthone was the variable, a solution of hycanthone at pH7.0 was made at a concentration of 16 mg/ml. This was diluted serially by half, six times, so that the final concentrations of the hycanthone solutions were 16 mg/ml, 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5mg/ml, 0.25 mg/ml. IA-4 solution was initially made a concentration of 2.0 mg/ml, and subsequently at 4.0 mg/ml.

4. Buffers

Phosphate buffers were made according to the methods outlined in *Methods in Enzymology*, Volume I, using 0.2M monobasic solution of potassium phosphate, and 0.2M solution of dibasic potassium phosphate.

For pH 5.9, 90.0 ml of the monobasic solution was added to 10.0 ml of the dibasic solution and diluted to 200 ml. For the pH 7.0 buffer, 39.0 ml of the monobasic solution was added to 61.0 ml of the dibasic solution and diluted to 200 ml.

If any adjustment was needed, it was done using the two phosphate solutions and not with acid or alkali.

Standard Lab Buffer is a 1/15 M solution of monobasic potassium phosphate. Its pH is approximately 4.7 and it is used for washing cell suspensions after treatment with the two compounds being tested.

B. Methods

1. Experimental Conditions

Because of the similarity of the structure of the hycanthone and the IA-4 to acridines, i.e. a three ring planar structure, any possible photodynamic effects were avoided by performance of the experiments in subdued lighting. For time-variation dose-action studies, the treated samples of yeast were kept in a dark cupboard and samples were removed when needed. For concentration-variation dose-action studies, the experiment was set up in subdued lighting, and kept in the aforementioned dark cupboard for the total time of treatment. All plating was done in dim light and all plates were incubated at 26.0 ± 0.5 degrees in the dark.

A study was made of the effect of light on the hycanthone-treated yeast. The bright light source was a New Brunswick Scientific incubator-shaker equipped with fluorescent lights having an energy of 1500 lux. Simultaneously, an experiment was done in continuous room lighting, and in the dark conditions described above.

All experiments were done at room temperature, which was approximately 26 degrees.

2. General Experimental Procedures for Dose-Action Studies

For dose-action studies, either with fixed drug concentration and variable time of exposure, or with fixed time of exposure and variable concentration, the methods used were generally the same.

The yeast cells were grown for three days on solid YEPD, i.e. until stationary phase, and were then scraped from the plates with sterile wooden spatulas, and suspended in sterile water. The concentration of the cells was adjusted to approximately 1×10^9 cells per ml. The suspension was left overnight under refrigeration, and recounted immediately before use. Any final adjustments in cell number were made at that time.

Of the cell suspension, 0.875 ml was added to a series of test-tubes each containing 0.125 ml of the hycanthone solution, the IA-4 solution, or buffer at the appropriate pH, so that each test-tube constituted one sample.

When time of exposure of the yeast cells to the compound in question was over, 9.0 ml of cold 1/15M buffer was added to those samples to be plated. This was vortexed to ensure mixing of the cells and the buffer, the level of the buffer at the bottom of the meniscus was marked on the test-tubes and they were spun down at 5,000 rpm in an IEC centrifuge in head number 269, for 20 minutes.

The solution was decanted out of the test-tubes and the yeast cells were washed a second time by adding the 1/15 M buffer up to the marked levels on the test-tubes, and were centrifuged a second time, for 20 minutes. The solutions were decanted and the level of buffer was again brought up

to the marks on the test-tubes, i.e. 10 mls.

A dilution series was done on each sample and 0.5 ml of a dilution of 10^5 was plated on each of four plates of YEPD. The undiluted samples were plated, 0.5 ml on each of four plates of -lys, -his, -thr, for strain XV169-15A. A 100-fold dilution was plated on four plates of -arg for X841.

The dilution of 10^5 gave the survival data. For all dose-action studies, four replicates were plated of every kind of media needed for reversion and survival data, so that all colony counts presented in the tables are a sum from four plates; i.e. per two ml.

This was the general procedure for all dose-action studies on both strains; any special conditions will be mentioned in conjunction with the individual experiments.

3. Gamma-Radiation

The irradiation was done using a ^{60}cobalt source (AECC) at a rate of 4.1 Krad per minute. For all experiments eight plates of media were used. For the strain XV169-15A, the cell suspension was adjusted to a concentration of 1×10^8 cells per ml and was irradiated after plating on YEPD at a dilution of 10^5 for survival, and at 0 dilution for reversion on -lys, -his, -thr.

For X841, the cell suspension was also adjusted to 1×10^8 cells per ml, and was irradiated after plating on YEPD at a dilution of 10^5 for survival, and at a dilution of 10^2 for the prototrophy on -arg.

RESULTS

All tables presented in Results are summarized computations of frequencies. The corresponding colony counts may be found in the Appendix under the corresponding table number, e.g. the actual colony counts from which Table 1 was calculated are given in Table A1 in the Appendix.

A. Mutagenicity and Toxicity of Hycanthone at pH 7.0 and pH 5.9 and of IA-4 at pH 5.9

I. The mutagenicity of hycanthone was tested at pH 7.0 because preliminary tests had shown that this is the pH at which the chemical is most active mutagenically. IA-4 does not dissolve above pH 6.0, therefore experiments using this compound were conducted at pH 5.9 and a comparable study was performed using hycanthone as a control at pH 5.9. The results are summarized in Tables 1 and 2. The actual colony counts are included in Tables A1 and A2 (see Appendix).

The yeast cells were treated at a final concentration of 0.125 mg/ml of hycanthone or of 0.25 mg/ml of IA-4, and samples were taken at the timed intervals indicated in column 1. A new parameter, ED, is also given in the table. It is the effective dose, being defined as the product of treatment time in hours multiplied by the dose in mg/ml. The fraction surviving is calculated as the percentage of the 0 time reading of the buffer controls. The reversion frequencies for *his1-7*, *lys1-1*, and *hom3-10* are expressed as revertants per survivor. All values shown have been corrected for the spontaneous mutations in the background by subtraction of the values found for the controls.

TABLE 1. Mutagenicity and toxicity of hycanthone at two pH's

pH	Treatment Time (Hours)	E.D.* (time x conc.)	Survival (% of control)	Reversion Frequencies			
				his 1-7 ($\times 10^6$)	*hom 3-10* ($\times 10^7$)	total *lys 1-1* ($\times 10^6$)	*lys 1-1* locus ($\times 10^8$)
5.9	0	0	98.5	-	-	0.05	1
	4.5	0.5625	87.1	0.49	-	0.15	2
	8	1.0	83.9	2.05	1.3	0.14	0
	12.5	1.5625	80.4	5.08	2.4	0.41	1
	22	2.75	71.5	22.6	4.6	1.84	6
	28	3.50	93.1	22.4	5.3	1.87	6
	34	4.25	68.1	38.6	6.8	2.63	12
	59	7.375	38.4	61.6	12.7	4.90	24
7.0	0	0	89.9	-	0.01	0.05	1
	4.5	0.5625	80.2	2.61	1.4	0.31	0
	8	1.00	86.7	6.09	4.4	0.17	1
	12.5	1.5625	75.4	15.3	7.2	1.26	5
	22	2.75	50.6	43.5	12.7	1.80	-
	28	3.50	37.5	43.0	15.3	1.80	7
	34	4.25	29.2	46.4	12.3	1.83	11
	59	7.375	9.9	33.9	5.5	2.83	7

*E.D. = effective dose, defined in text.

TABLE 2. Mutagenicity and toxicity of IA-4 at pH 5.9

Treatment Time (Hours)	E.D.* (time x conc.)	Survival (% of control)	Reversion Frequencies			
			his 1-7 (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)	*lys 1-1* locus (x 10^8)
0	0	81.2	-	-	-	1
4.5	0.5625	-	-	-	-	-
8	1.00	80.0	-	-	-	-
12.5	1.5625	85.2	.14	-	-	-
22	2.75	69.5	-	-	-	-
28	3.50	-	-	-	-	-
34	4.25	68.0	-	.10	-	1
59	7.375	60.1	-	1.02	-	-

"-" indicates less than control.

The toxicity of hycanthone at two different pH's and of IA-4 is shown in Figure 2. It can be seen that hycanthone is more toxic to the yeast at pH 7.0 than at pH 5.9, whereas IA-4 is less toxic than hycanthone under the given conditions.

The mutagenicity of hycanthone at pH 7.0 and pH 5.9 for reversions at the *his1-7* locus are shown in Figure 3. The rate of reversion of *his1-7* at these two pH's, as shown by the parallel slopes of the lines, appears to be approximately the same; the overall mutation frequency at any given time of exposure at pH 7.0 is about twice that found at pH 5.9. The reversion patterns for *hom3-10* after treatment with hycanthone at pH 7.0 and pH 5.9 are shown in Figure 4. Again the reversion rates are about the same for the two pH's, but the frequency of revertants at a given concentration is two to three times greater at pH 7.0 than at pH 5.9.

The *lys1-1* reversion patterns for hycanthone at pH 7.0 and pH 5.9 are shown in Figure 5. This graph reveals that there is no differential mutagenic action of hycanthone on *lys1-1* at pH 7.0 and pH 5.9. The data plotted for the *lys1-1* are the sum of mutations induced at the supersuppressors and at the *lys1-1* locus. The frequencies of reversions of locus itself are shown in the last column of the tables. It can be seen that the reversions at the locus increase with increasing exposure to hycanthone, at pH 7.0 and at pH 5.9.

In all the figures, it can be seen that the slopes of the lines for *lys1-1* and *hom3-10* reversions are approximately one, whereas the slope of *his1-7* reversion is approximately two.

Table 2 shows that IA-4 is not mutagenic for any of the markers used and under the given experimental conditions.

Figure 2. Survival of yeast after different hours of exposure to hycanthone at pH 7.0 and pH 5.9 and to IA-4 at pH 5.9.

● Hycanthone pH 7.0

○ Hycanthone pH 5.9

□ IA-4 pH 5.9

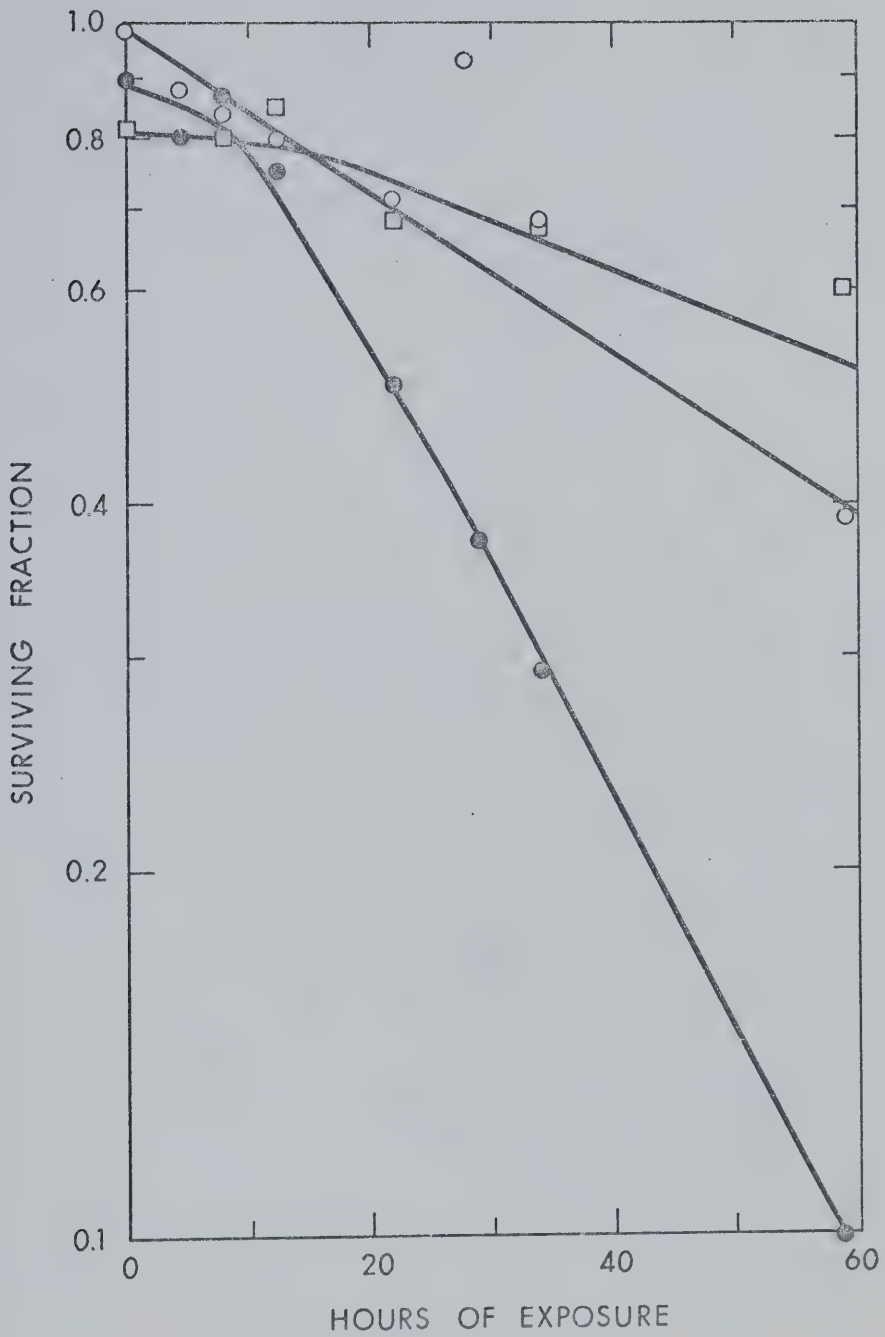

1.0
0.8
0.6
0.4
0.2
0.1
SURVIVING FRACTION
0
20
40
60
HOURS OF EXPOSURE

Figure 3. Reversion frequencies of *his 1-7*, after different hours of exposure to hycanthone at pH 7.0 and pH 5.9.

○ Hycanthone pH 7.0

● Hycanthone pH 5.9

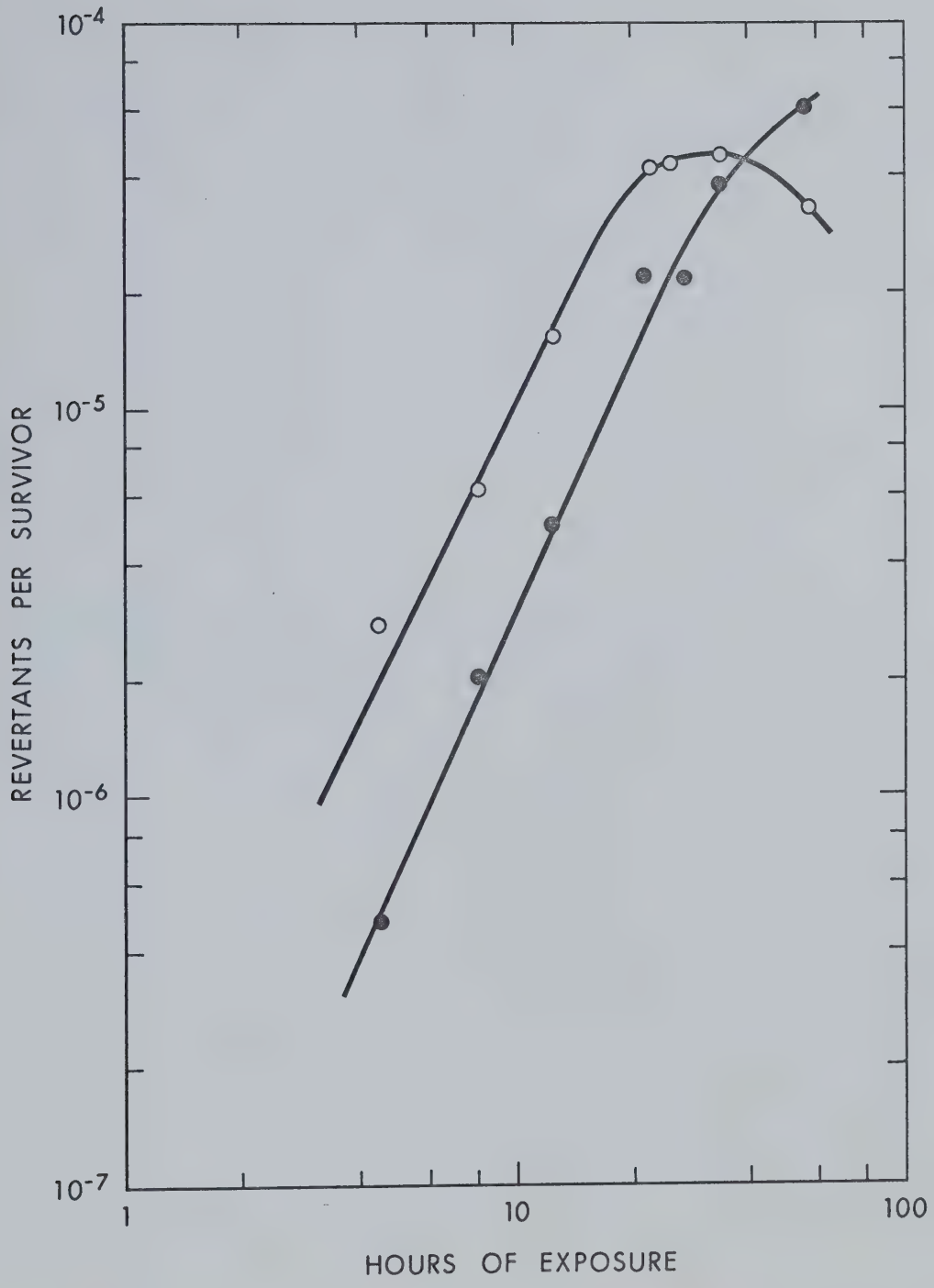

10^{-4}
10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

Figure 4. Reversion frequencies of *hom 3-10*, after different hours of exposure to hycanthone, at pH 7.0 and pH 5.9.

○ Hycanthone pH 7.0

● Hycanthone pH 5.9

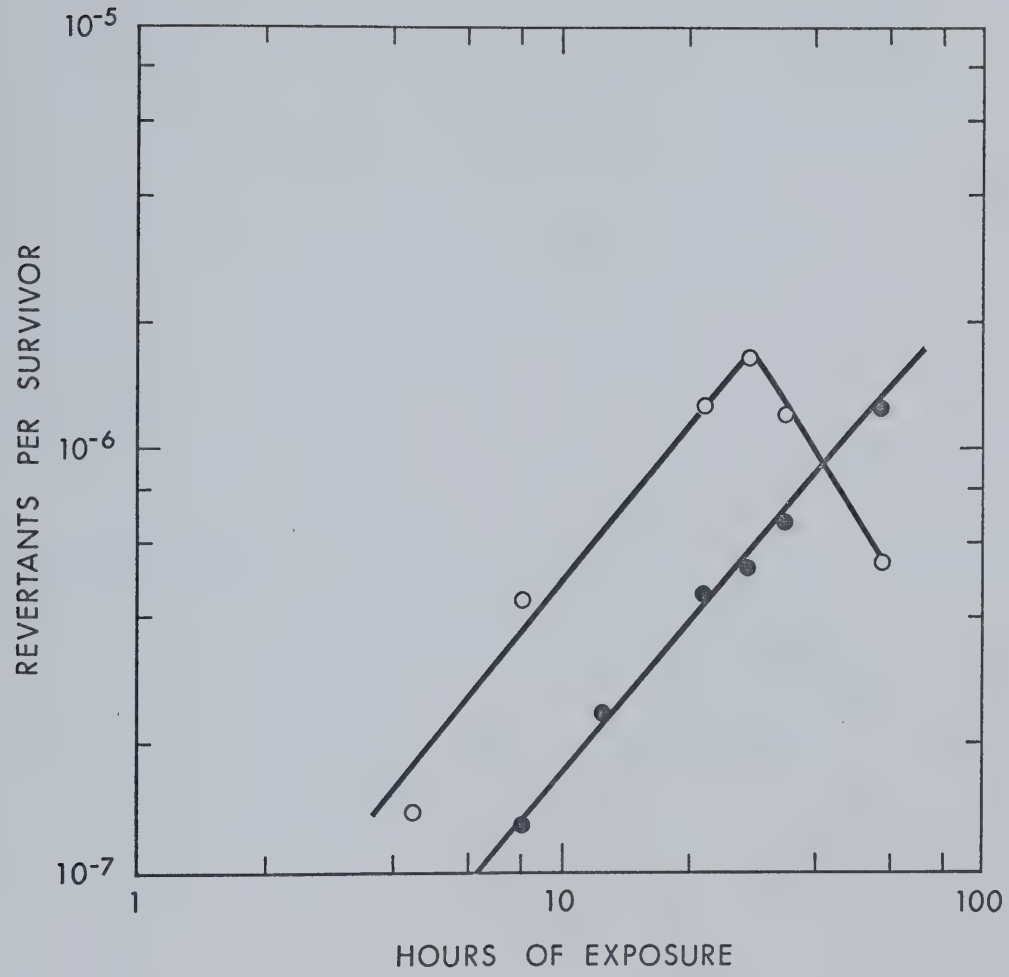
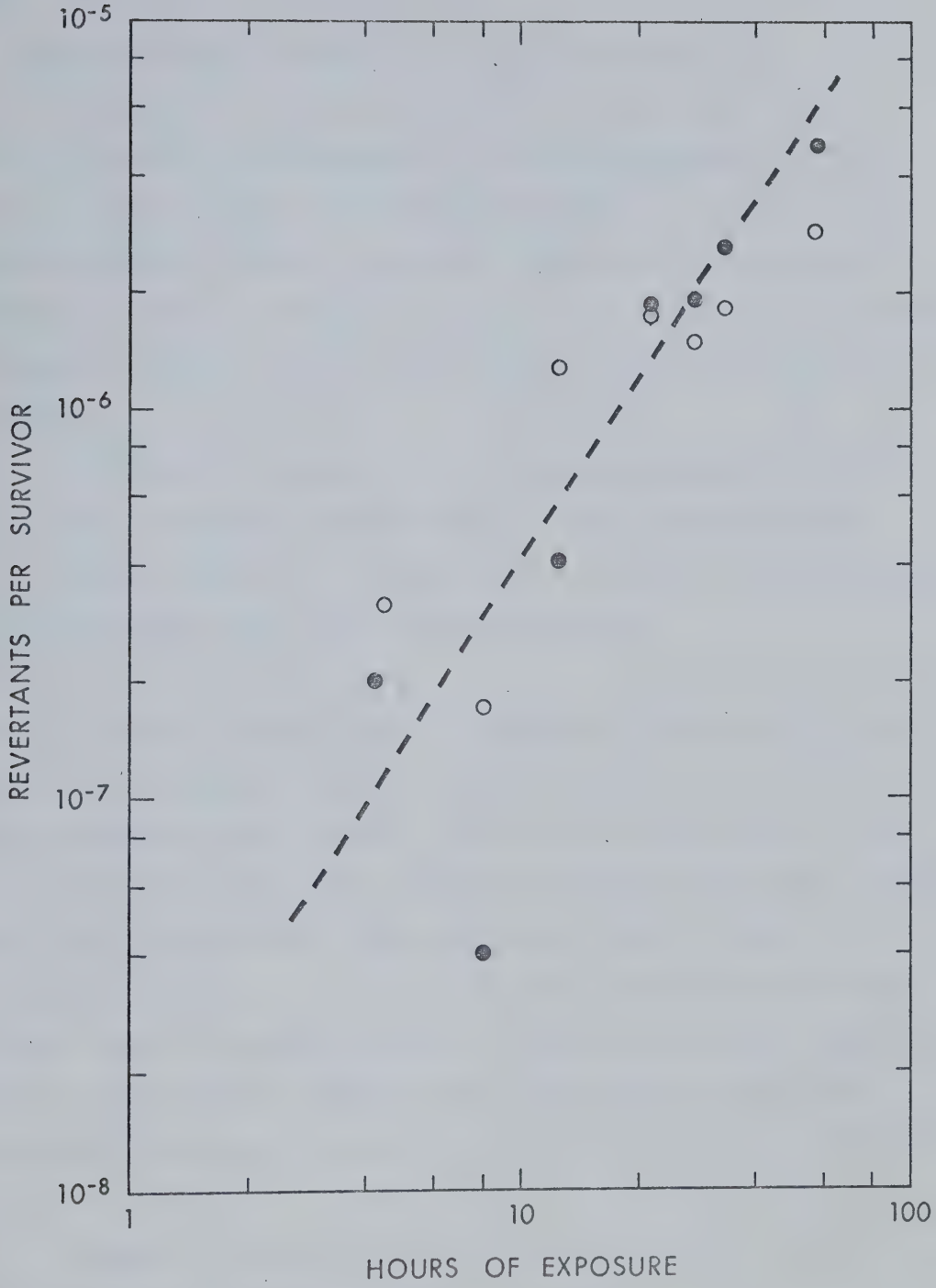

10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

Figure 5. Reversion frequencies of *lys 1-1*, after different hours of exposure to hycanthone, at pH 7.0 and pH 5.9.

○ Hycanthone pH 7.0

● Hycanthone pH 5.9


10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

II. The treatment of the yeast with hycanthone at pH 7.0 was repeated to verify the results obtained in the first experiment.

The results of the three experiments are presented in Table 3. The data from all three experiments are plotted graphically in Figures 6, 7, 8, and 9. It can be seen that the results of hycanthone toxicity and mutagenicity at pH 7.0 are generally reproducible. The mutation frequency of *his1-7* again gives a slope of approximately two. The mutation frequency reaches a maximum at about 30 hours of exposure, after which it declines.

The reversion frequency of *hom3-10* again demonstrates a slope of one, and it too reaches a peak, at about 25 hours, and then declines.

Lys1-1 demonstrates a slope of one, but does not decline as do the two other markers after 25 to 30 hours of exposure.

III. Since IA-4 was not found to be mutagenic in yeast at the concentration of 0.250 mg/ml at pH 5.9, the IA-4 therefore was increased to a final concentration of 0.5 mg/ml. The yeast cells were treated at this concentration at pH 5.9, and a parallel experiment was run with hycanthone at a final concentration of 0.125 mg/ml, at pH 5.9. The results are presented in Tables 4, 5, and 6. The control values have not been subtracted from the reversion frequencies obtained in the treated samples as even at this four-fold increase in IA-4 concentration compared with hycanthone, there is no increase in mutation frequency above the background level.

However, at this increased concentration, IA-4 appears to be very toxic to the yeast. Only 57.1% of yeast

TABLE 3. Action of hycanthone at pH 7.0 in three experiments

Experiment	Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies *his 1-7* (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)
I	0	108.2	-	-	-
	7	100.4	7.84	5.18	3.60
	18	53.9	37.14	15.74	8.70
	24	48.2	34.81	11.29	9.04
	27	38.8	35.01	13.22	9.06
	30	31.2	44.88	13.74	11.04
	33	25.5	54.46	11.27	24.13
	55	9.5	58.55	16.96	28.64
II	0	102	0.035	0.19	-
	6.5	103.2	1.86	1.24	0.92
	16.5	90.7	10.41	4.74	3.83
	18	77.9	14.83	5.67	8.62
	20	82.1	20.86	6.46	-
	23	57.3	25.24	11.84	-
	25.5	40.7	29.33	22.62	-
	30.5	28.1	36.12	18.54	-
	51	6.5	12.24	10.51	-
III	0	104.5	-	0.36	0.66
	12	100.2	7.81	5.89	3.90
	18	84.3	21.78	6.25	6.49
	22	53.6	28.02	14.13	8.34
	24	54.5	29.48	11.08	9.54
	48	24.9	15.19	5.39	8.04
	66	14.7	21.04	6.29	22.87

Figure 6. Survival of yeast after different hours of exposure to hycanthone, at pH 7.0, for three experiments.

○ Experiment I

● Experiment II

△ Experiment III

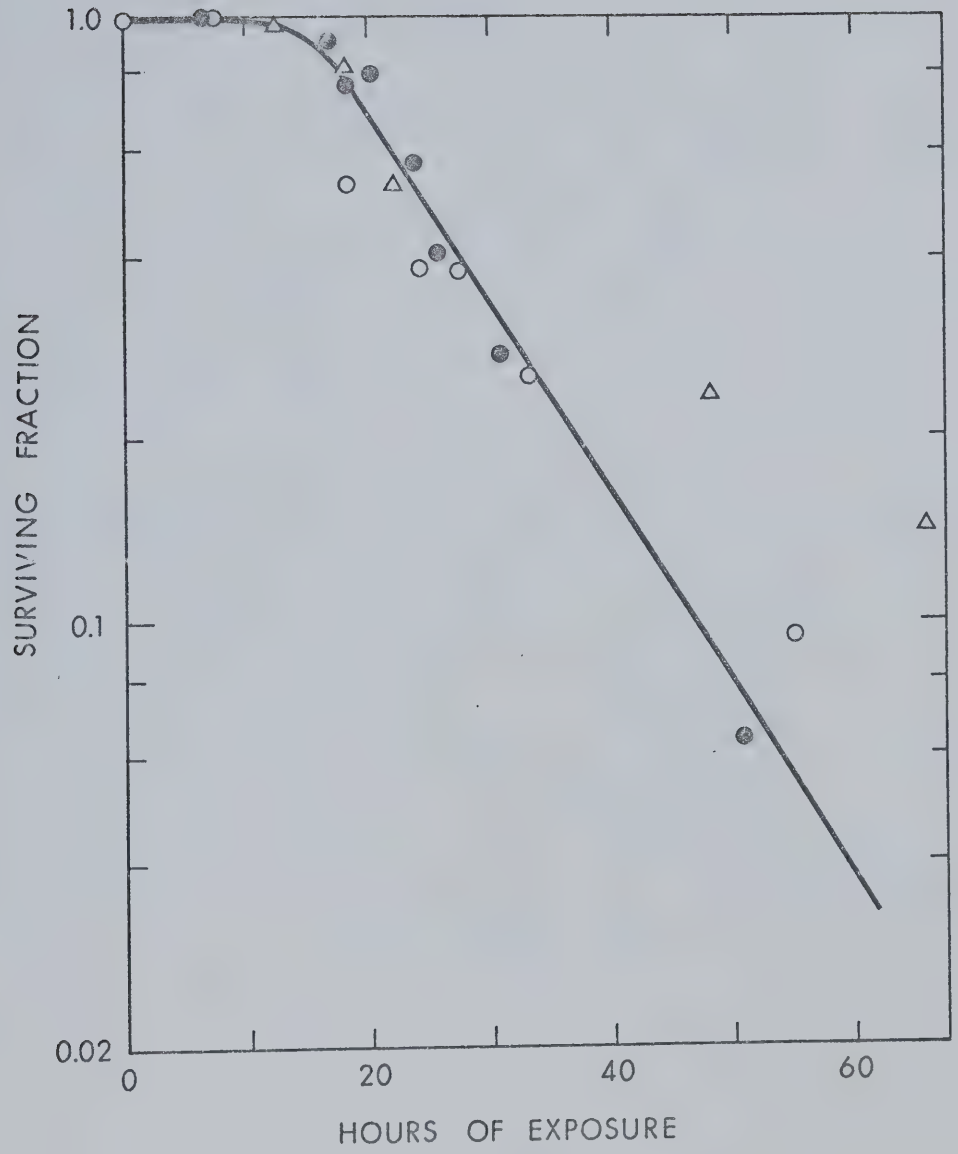

1.0
0.1
0.02
SURVIVING FRACTION
0
20
40
60
HOURS OF EXPOSURE

Figure 7. Reversion frequencies of *his 1-7*, after different hours of exposure to hycanthone at pH 7.0, for three experiments.

○ Experiment I

● Experiment II

△ Experiment III

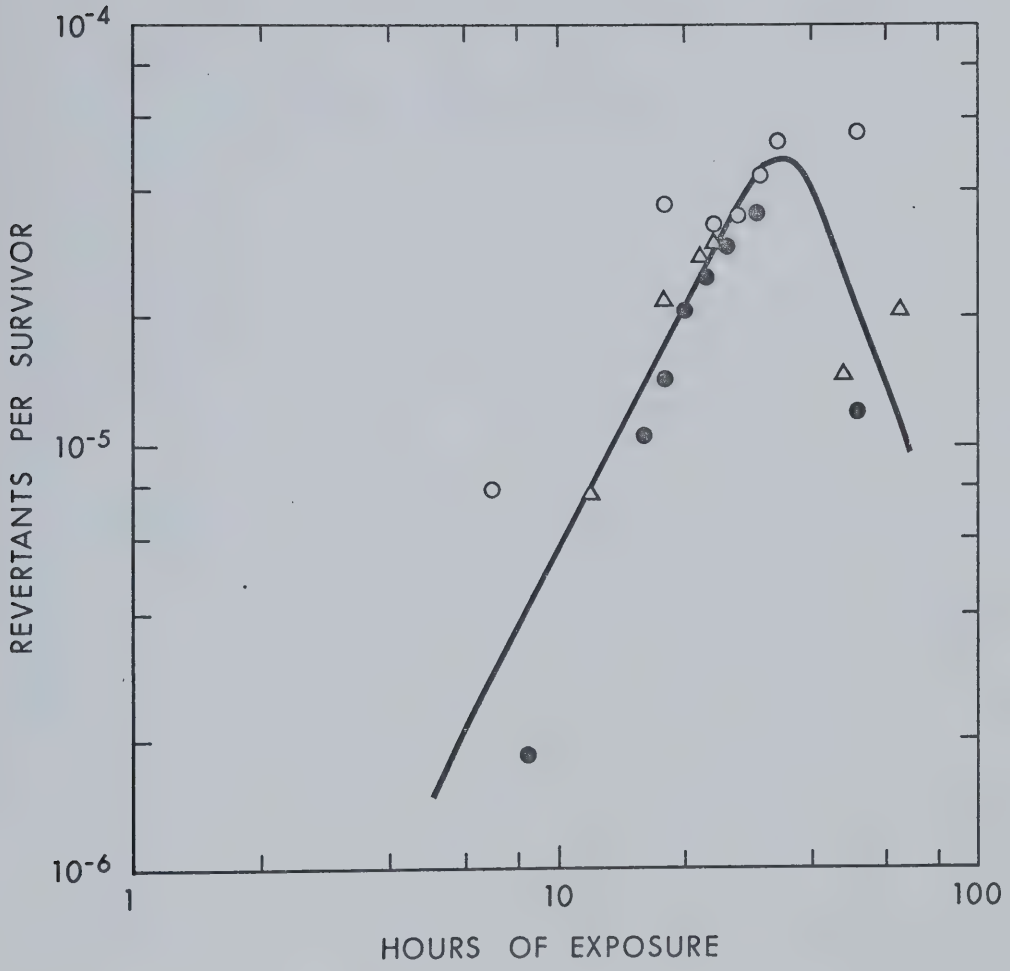

10^{-4}
10^{-5}
10^{-6}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

Figure 8. Reversion frequencies of *hom 3-10*, after different hours of exposure to hycanthone at pH 7.0, for three experiments.

○ Experiment I

● Experiment II

△ Experiment III

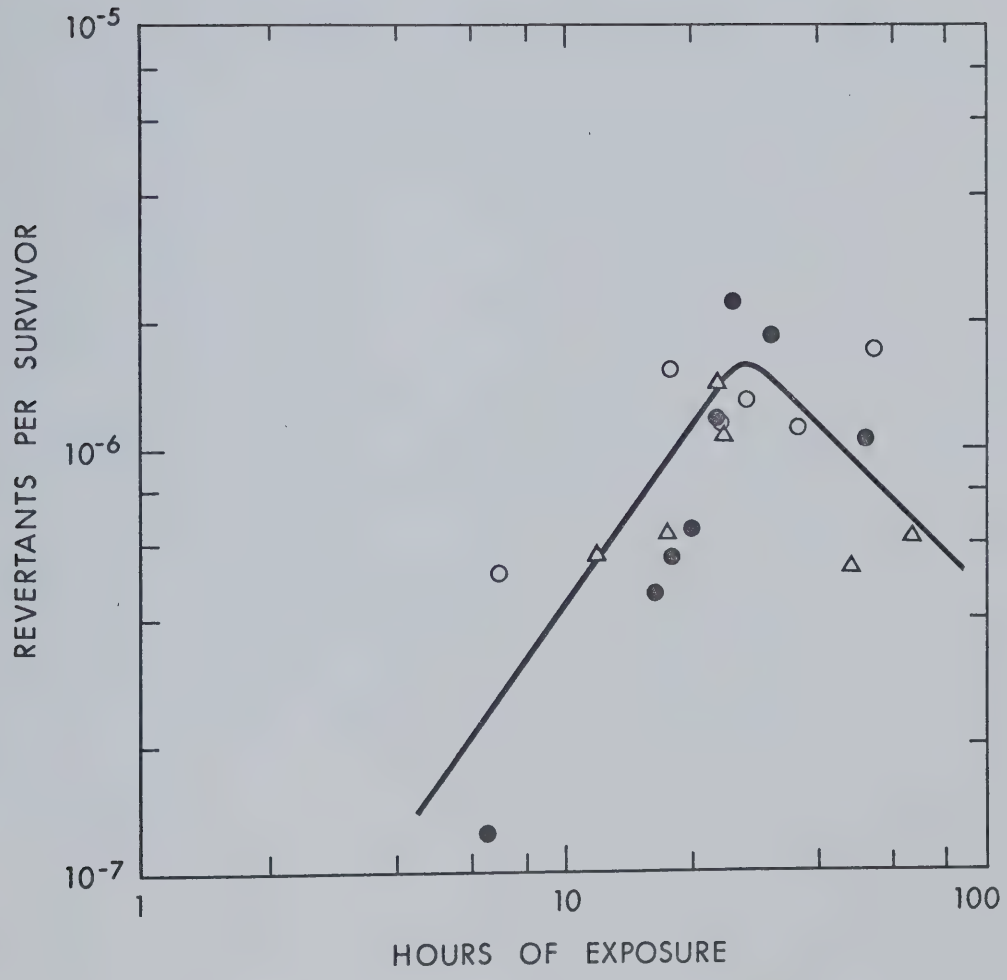

10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

Figure 9. Reversion frequencies of *lys 1-1*, after different hours of exposure to hycanthone at pH 7.0, for three experiments.

○ Experiment I

● Experiment II

△ Experiment III

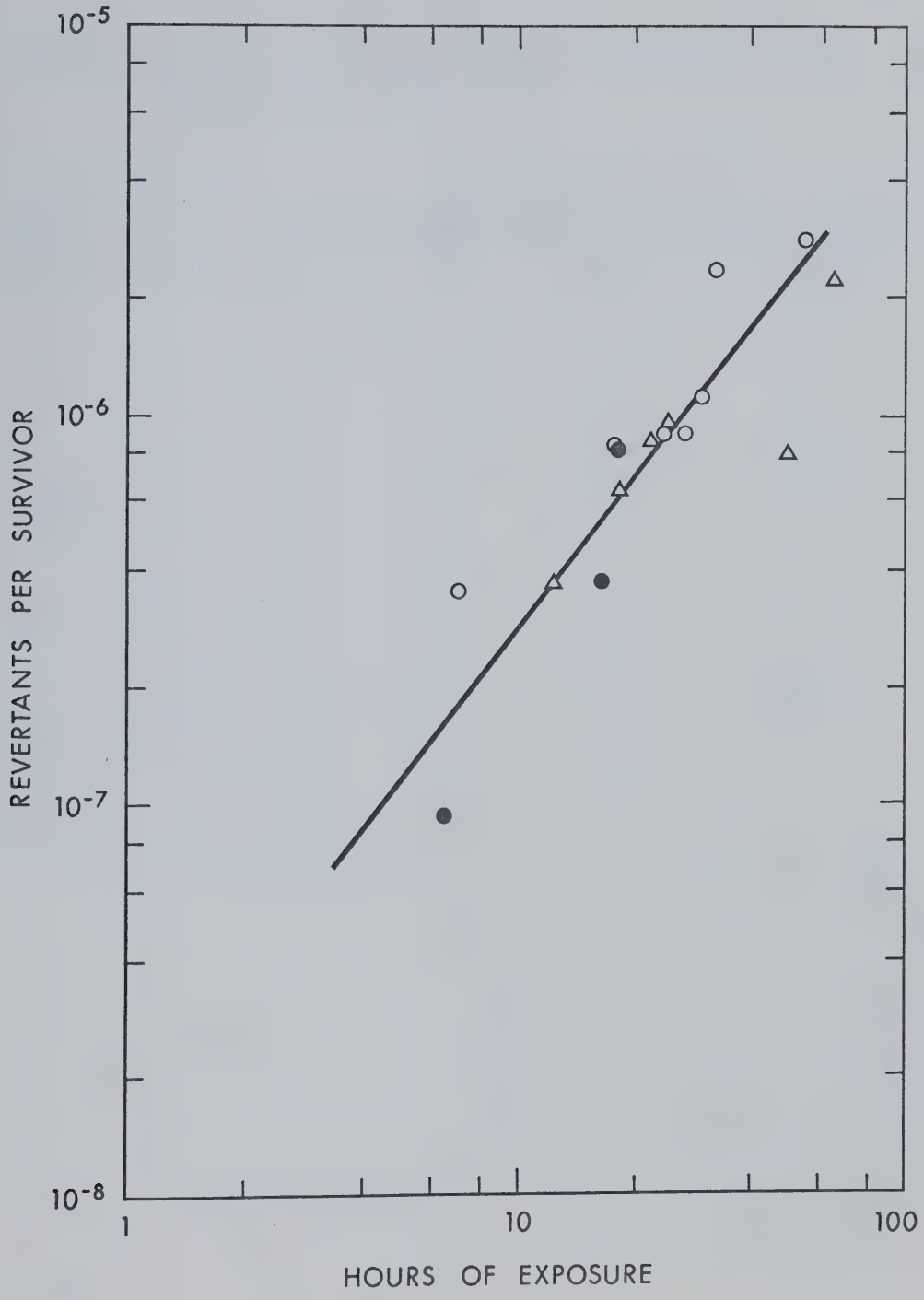

10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

TABLE 4. Surviving fractions and reversion frequencies of yeast in control buffer pH 5.9

Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies			
		his 1-7 (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)	*lys 1-1* locus (x 10^8)
0	100	1.28	0.27	0.80	2.72
8	100	1.31	0.20	0.89	0
16	99.9	1.41	0.41	0.80	1.36
25.5	97.4	1.39	0.14	0.85	1.40
35.5	83.4	1.69	0.33	1.06	0
46	100	1.25	0.64	0.85	0
60	92.4	1.54	0.44	0.96	0
71	90.6	1.41	0.60	0.91	1.50
112	95.0	1.38	0.36	0.77	0
161	89.8	1.08	0.23	0.81	0

TABLE 5. Surviving fractions and reversion frequencies of yeast treated with hycanthone at pH 5.9

Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies			
		his I-7 (x 10^6)	*hom 3-I0* (x 10^7)	total *lys I-I* (x 10^6)	*lys I-I* locus (x 10^8)
0	108.4	1.17	0.31	0.77	0
8	98.4	2.97	1.45	0.96	0
16	95.9	4.81	2.20	0.94	2.13
25.5	73.1	9.22	2.51	1.19	2.13
35.5	56.2	17.52	6.06	1.58	4.85
46	42.3	27.69	9.00	2.54	14.47
60	37.6	23.56	4.53	2.37	14.49
71	21.8	25.34	5.63	2.97	21.88
112	10.2	36.14	6.00	4.27	6.67
161	5.7	24.04	8.41	8.47	12.05

TABLE 6. Surviving fractions and reversion frequencies of yeast treated with IA4 at pH 5.9

Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies			
		his 1-7 (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)	*lys 1-1* locus (x 10^8)
0	57.1	1.43	0.12	0.22	0
8	46.4	1.28	0.29	0.15	0
16	54.9	1.54	0.25	0.12	0
25.5	35.6	1.34	0.57	0.12	0
35.5	33.4	0.88	0.61	0.18	2.04
46	22.1	0.93	0.62	0.22	0
60	21.2	1.28	0.64	0.06	0
71	22.5	1.15	0.30	0.12	6.06
112	15.4	1.42	0	0.27	0
161	7.9	1.72	0	0	0

survive at the 0 time treatment with IA-4; nevertheless the IA-4 does not induce reversions in any of the three loci tested.

IV. To determine if the very toxic effect of IA-4 observed in the last experiment could be attributed to this compound not being properly washed out of the cells so that it continued to act on the yeast, even after plating, the experiment was repeated, using IA-4 again at 0.5 mg/ml final concentration. This time the yeast cells were washed four times, instead of twice, after the treatment.

The experiment was done twice. The results are given in Tables 7 and 8. Control values are not subtracted from the IA-4 and the hycanthone data. It can be seen that, even with the cells being washed four times, IA-4 is still very toxic to the yeast at 0.5 mg/ml. The toxicity is the same as after having washed the cells twice. On the other hand, hycanthone at pH 5.9 consistently induces reversions at all three loci studied, but IA-4 does not.

B. Mutagenicity and Toxicity of Hycanthone at Different Concentrations

Tables 9 and 10 show the effects of hycanthone used at varying concentrations at two different time intervals.

The hycanthone was dissolved in pH 7.0 at a concentration of 16 mg/ml and diluted serially by half as described in the MATERIALS and METHODS section. 0.125 ml of each solution was used to treat the yeast cells. The final concentration used is indicated in the first column of each table. From this, the effective dose, ED, is calculated as mentioned earlier, by multiplying the dose in mg/ml by the time in hours of exposure of the yeast to hycanthone.

TABLE 7. Surviving fractions and reversion frequencies of yeast treated with buffer, pH 5.9, and hycanthone at final concentration of 0.125 mg/ml and IA4 at final concentration of 0.5 mg/ml, and washed four times. (Exp. I)

	Treatment Time (Hours)	Survival (% of control	Reversion Frequencies *his 1-7* (x 10^6)	*hom 3-10* (x 10^7)	*lys 1-1* (x 10^6)
Buffer pH 5.9	0	100	1.39	0.44	0.78
	12	106.9	1.21	0.25	0.79
	24	106.5	1.03	0.66	0.97
	48	104.9	0.91	0.34	0.67
	66	96.9	1.19	0.27	0.71
Hycanthone pH 5.9	0	103.9	1.41	0.34	0.89
	12	92.7	5.11	1.81	1.12
	24	91.6	13.15	5.29	1.54
	48	31.8	33.96	9.42	2.52
	66	12.0	43.02	25.00	2.35
IA4 pH 5.9	0	64.1	1.17	0.14	0.10
	12	35.0	1.06	0.76	0.20
	24	36.0	1.15	0.25	0.10
	48	23.6	1.05	0	0.08
	66	19.8	2.84	14.22	0.71

TABLE 8. Surviving fractions and reversion frequencies of yeast treated with buffer, pH 5.9, and hycanthone at final concentration of 0.125 mg/ml and IA4 at final concentration of 0.5 mg/ml, and washed four times. (Exp. II)

	Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies *his 1-7* (x 10^6)	*hom 3-10* (x 10^7)	*lys 1-1* (x 10^6)
Buffer pH 5.9	0	100	1.04	0.73	2.25
	9.5	96.8	0.87	0.43	2.68
	24	100.9	0.88	0.83	2.50
	33.5	100.7	0.84	0.42	2.67
	54	95.2	0.95	0.55	2.60
	73	100.0	0.92	0.42	2.69
Hycanthone pH 5.9	0	98.5	0.84	0.75	2.42
	9.5	100.7	2.14	1.25	2.63
	24	84.4	7.98	2.86	2.83
	33.5	64.6	24.68	6.82	4.03
	54	32.5	30.00	15.76	4.34
	73	13.1	22.16	9.60	5.04
IA4 pH 5.9	0	56.5	0.83	0.37	0.13
	9.5	38.3	0.74	0.54	0.06
	24	22.9	0.96	0.46	0.05
	33.5	22.0	1.38	0	0.05
	54	11.5	0.36	0	0
	73	9.1	1.26	0	0

TABLE 9. Mutagenicity and toxicity of hycanthone at different concentrations on yeast, after 8 hours of treatment

Hycanthone mg/ml	E.D.	Survival (% of control)	Reversion Frequencies			
			his 1-1 (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)	*lys 1-1* locus (x 10^8)
0	0	100	0	0	0	0
0.031	.248	100	0.36	0	-	-
0.062	.496	90.1	1.51	0.57	0.38	1.93
0.125	1.0	89.6	4.02	2.24	0.21	2.88
0.250	2.0	93.5	6.30	3.11	0.34	0.95
0.5	4.0	65.1	10.34	5.70	0.50	4.27
1.0	8.0	43.3	10.20	3.09	-	3.00
2.0	16.0	21.7	4.49	3.28	-	3.00

TABLE 10. Mutagenicity and toxicity of hycanthone at different concentrations, on yeast, after 12 hours of treatment

Hycanthone mg/ml	E.D.	Survival (% of control)	Reversion Frequencies			
			his 1-1 (x 10^6)	*hom 3-10* (x 10^7)	total *lys 1-1* (x 10^6)	*lys 1-1* locus (x 10^8)
0	0	100	0	0	0	0
0.031	0.372	98.4	0.51	0.08	0.29	0
0.062	0.744	87.7	2.10	0.70	0.20	0
0.125	1.500	82.0	6.22	3.11	0.60	2.29
0.250	3.00	70.6	12.89	6.48	0.63	1.89
0.50	6.00	59.1	17.70	9.72	0.53	5.07
1.00	12.00	42.2	5.83	6.70	0.20	1.24
2.00	24.00	15.4	3.00	4.72	0.23	2.49

The surviving fractions of yeast cells after exposures of 8 hours and 12 hours are shown in Figure 10. As would be expected, the longer treatment time enhances the killing rate of hycanthone.

Figures 11, 12, and 13 show the mutagenic effect of hycanthone on the *his1-7*, the *hom3-10*, and the *lys1-1* markers respectively. The overall frequencies for the three markers are higher at 12 hours of exposure than at 8 hours of exposure. It can also be seen that the mutation frequencies for *his1-7* and *hom3-10* increase with increase of hycanthone concentrations. However, this is not the case for *lys1-1*. At the two different exposure times used, the mutation frequency of *lys1-1*, over the range of concentrations used, does not increase significantly above the background reversion, which is indicated by the "spontaneous" line. The reversion frequencies for *his1-7* and *hom3-10* each maximize at 0.5 mg/ml for both 8 hour and 12 hour treatments.

C. Relative Toxicity and Mutagenicity of Hycanthone at pH 7.0 Under Dark and Light Conditions

Since hycanthone has a three ring planar structure, similar to that of acridines, which are known to show photodynamic action in the light, hycanthone was tested in three different lighting conditions, dark, room light and bright light and the results are given in Table 11.

For the dark experiment, except for plating which was done under dim light, the experiment was kept in the dark and plates were incubated in the dark. A parallel experiment was done in continuous room lighting (the light energy was not measured), and a third experiment was performed under the bright fluorescent lights with energy of 750 lux of incident exposure.

Only reversion frequencies for *his1-7* and *hom3-10* are given in the tables. The *lys1-1* data are not given here but can be found in the

Figure 10. Survival of yeast after exposure to different concentrations of hycanthone for eight hours and twelve hours.

● 8 hours of exposure

○ 12 hours of exposure

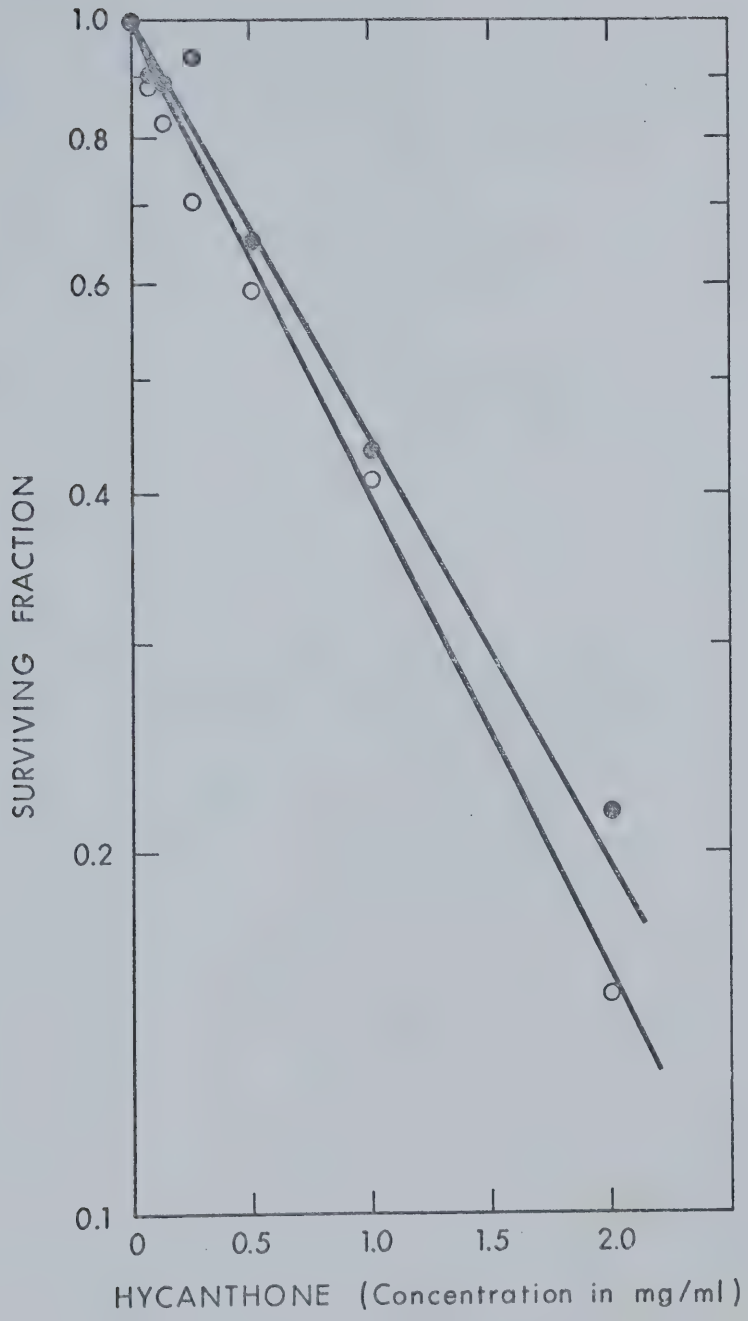

1.0
0.8
0.6
0.4
0.2
0.1
SURVIVING FRACTION
0
0.5
1.0
1.5
2.0
HYCANTHONE (Concentration in mg/ml)

Figure 11. Reversion frequencies of *his 1-7*, after treatment with different concentrations of hycanthone for eight hours and twelve hours.

● 8 hours of exposure

○ 12 hours of exposure

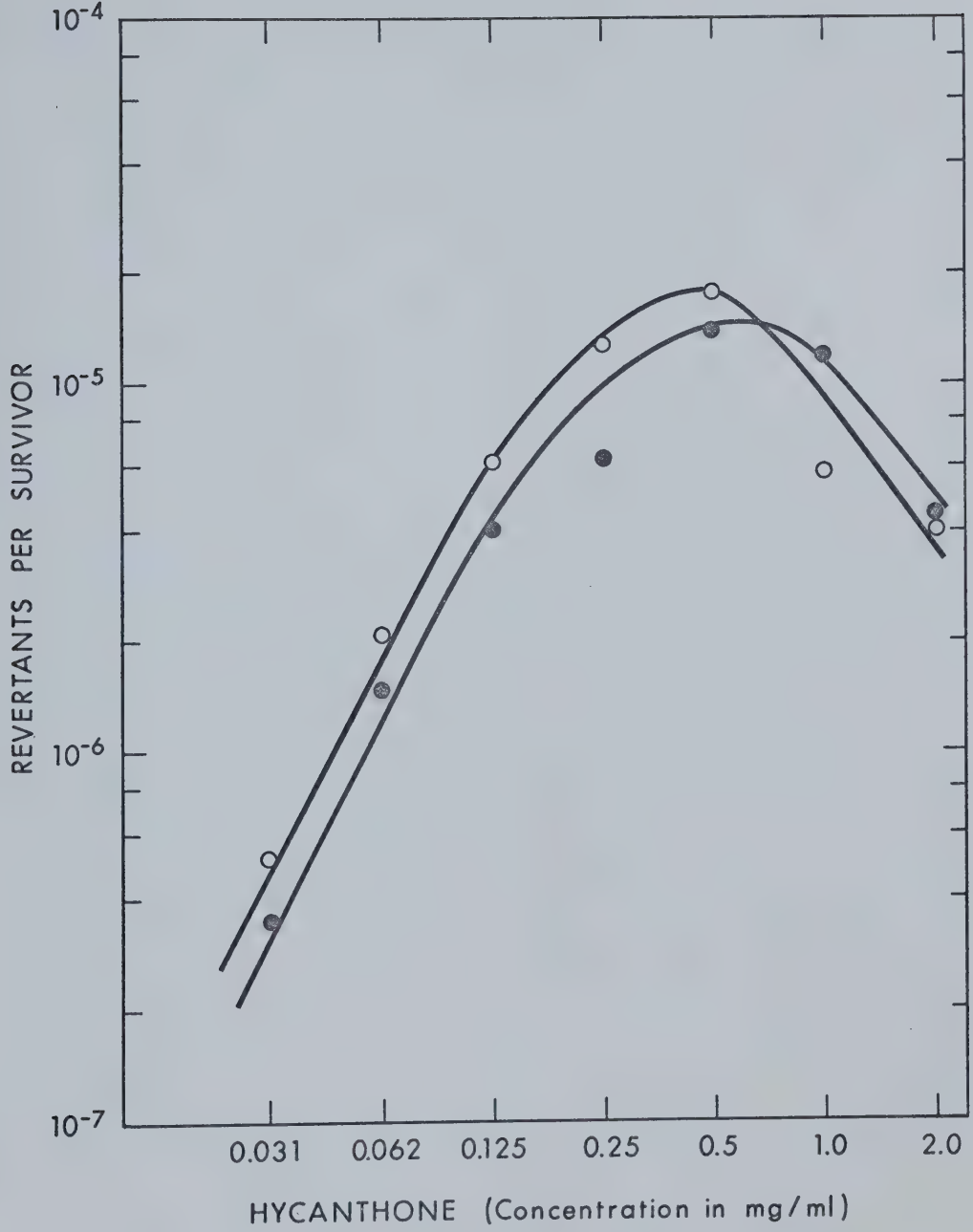

10^{-4}
10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
0.031
0.062
0.125
0.25
0.5
1.0
2.0
HYCANTHONE (Concentration in mg/ml)

Figure 12. Reversion frequencies of *hom 3-10*, after treatment with different concentrations of hycanthone for eight hours and twelve hours.

● 8 hours of exposure

○ 12 hours of exposure

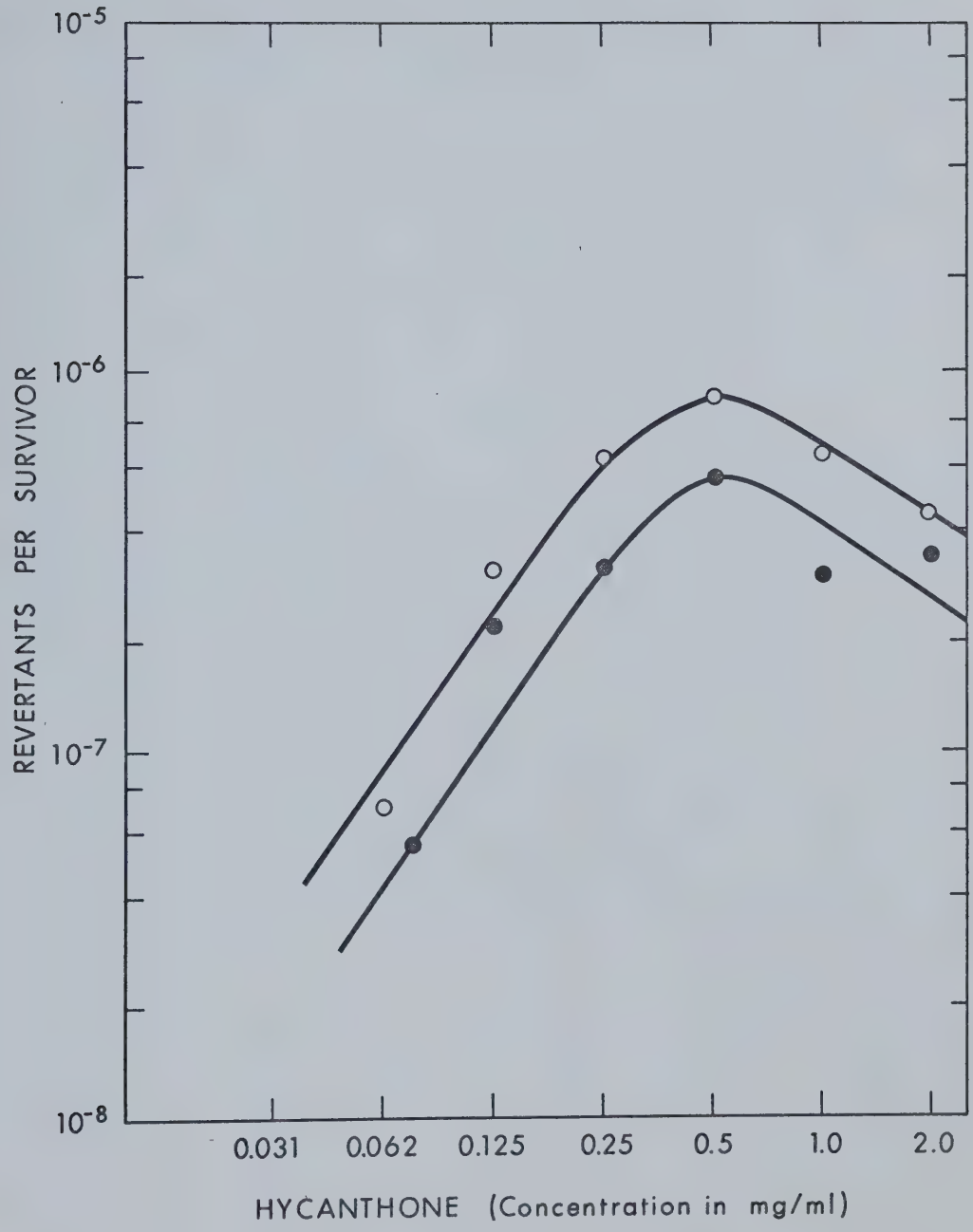

10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
0.031
0.062
0.125
0.25
0.5
1.0
2.0
HYCANTHONE (Concentration in mg/ml)

Figure 13. Reversion frequencies of *lys 1-1*, after treatment with different concentrations of hycanthone for eight hours and twelve hours.

● 8 hours of exposure

○ 12 hours of exposure

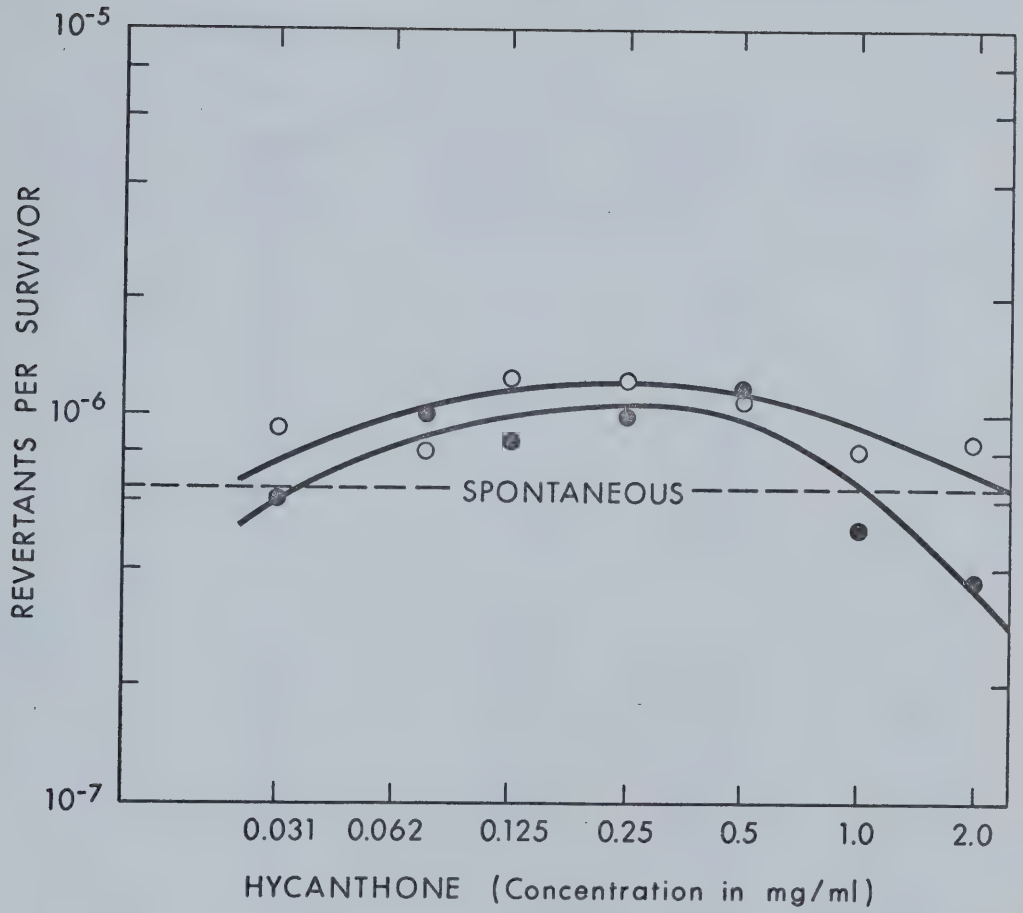

10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
SPONTANEOUS
0.031
0.062
0.125
0.25
0.5
1.0
2.0
HYCANTHONE (Concentration in mg/ml)

TABLE 11. Mutagenicity and toxicity of hycanthone at a final concentration of 0.125 mg/ml under various conditions of light

Conditions of Light	Treatment Time (Hours)	Survival (% of control)	Reversion Frequencies *his 1-7* (x 10^6)	Reversion Frequencies *hom 3-10* (x 10^7)
Darkness	0	92.5	0.26	0.03
	5.5	77.7	2.65	1.33
	9	82.8	4.47	3.55
	14.5	70.7	7.20	5.43
	17	64.0	7.86	4.13
	22	68.6	9.42	4.81
	34	39.0	29.62	12.81
Room lighting	0	101.8	-	.08
	5.5	91.2	3.44	2.84
	9	73.5	8.10	4.58
	14.5	72.3	11.25	5.67
	17	52.9	18.47	8.47
	22	43.7	22.85	12.54
	34	14.3	34.02	7.44
	54.5	2.9	13.69	10.15
Bright light	0	98.6	0.10	.01
	5.5	84.4	4.53	3.10
	9	71.2	9.53	6.31
	14.5	40.7	23.79	12.39
	17	32.6	31.30	17.66
	22	30.4	30.44	16.84
	34	5.0	35.80	42.10
	54.5	3.5	32.26	60.82

Appendix, Table A11. This is because *lys1-1* had an unexpectedly high background reversion frequency in this set of experiments.

Figure 14 shows that the survival of the yeast, when treated with hycanthone, is affected by the lighting conditions. The killing is greatest when the light is strongest, and least in the dark. Light also enhances the reversion frequencies for *his1-7* and *hom3-10*. Figure 15 shows the *his1-7* reversion frequency for the three different lighting conditions. There is about a 1.5 times enhancement of reversion frequency in room light compared to that in the dark. There is a further 1.5 times enhancement from room lighting to bright lighting; i.e. there is a total of about three-fold increase for *his1-7* reversions in bright light compared to that in the dark.

Figure 16 shows the *hom3-10* reversion pattern under the different light conditions. It is noted in this figure that *hom3-10* reversions continued to increase in the bright light, whereas in room light and in the dark the reversion begins to decline after 22 hours of exposure to hycanthone. This differs from *his1-7* reversion, where under all light conditions, a peak is reached at about 30 hours, after which the reversion frequency declines.

D. Gamma-Ray Lethality and Mutagenicity on Strain XV169-15A

In order to calibrate the mutagenicity of hycanthone to that of a known physical mutagen the action of hycanthone was compared to that of gamma-rays.

Table 12 gives the survival and the reversion frequencies of the three markers studied, after irradiation with the dose indicated. The data is presented graphically in Figures 17 and 18.

Figure 14. Survival of yeast after different hours of exposure to hycanthone, pH 7.0, under various lighting conditions.

○ hycanthone under dark conditions

● hycanthone under room lighting conditions

□ hycanthone under bright lighting conditions

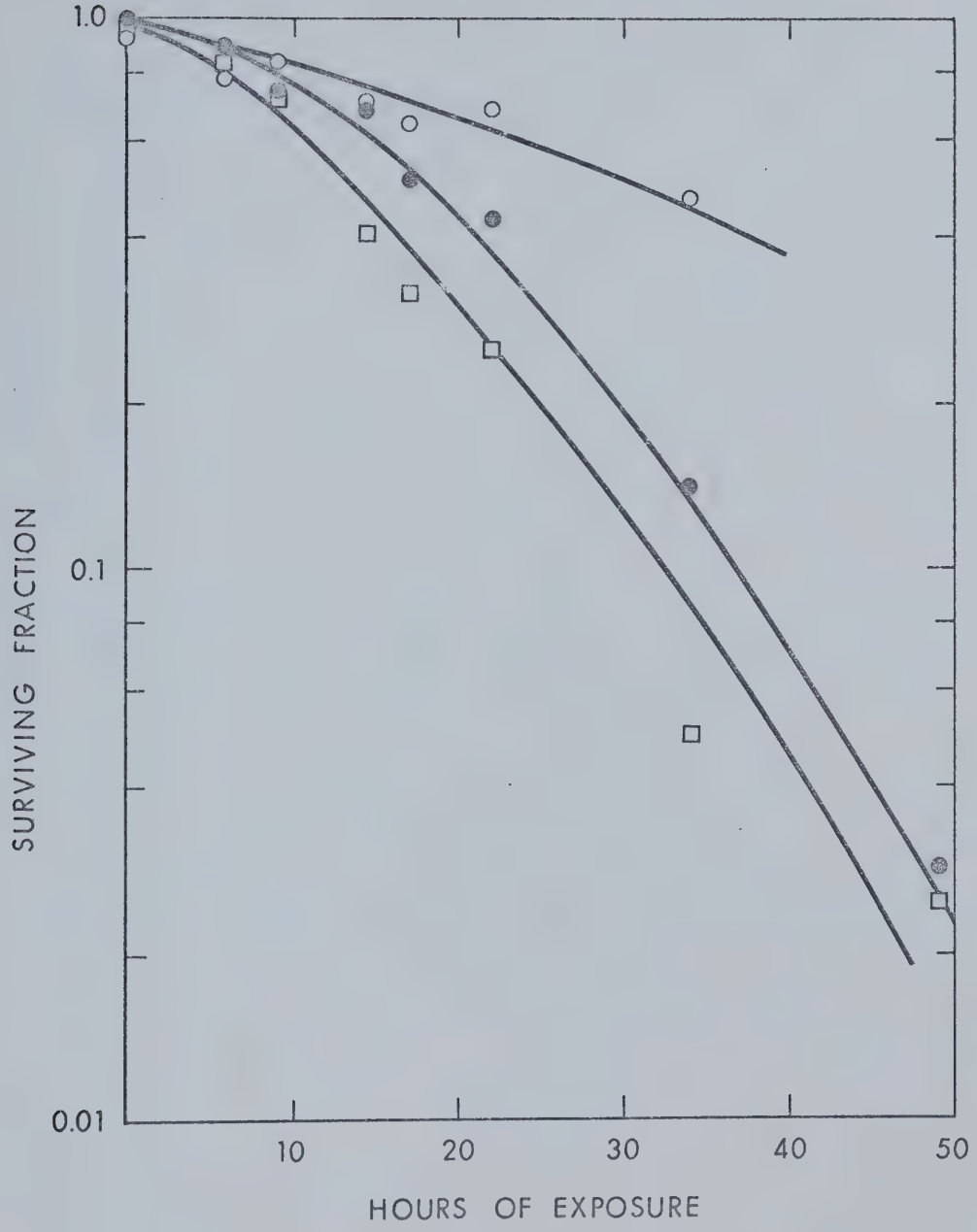

1.0
0.1
0.01
SURVIVING FRACTION
10
20
30
40
50
HOURS OF EXPOSURE

Figure 15. Reversion frequencies of *his 1-7* after different hours of exposure to hycanthone, pH 7.0, under various lighting conditions.

○ hycanthone under dark conditions

● hycanthone under room lighting conditions

□ hycanthone under bright lighting conditions

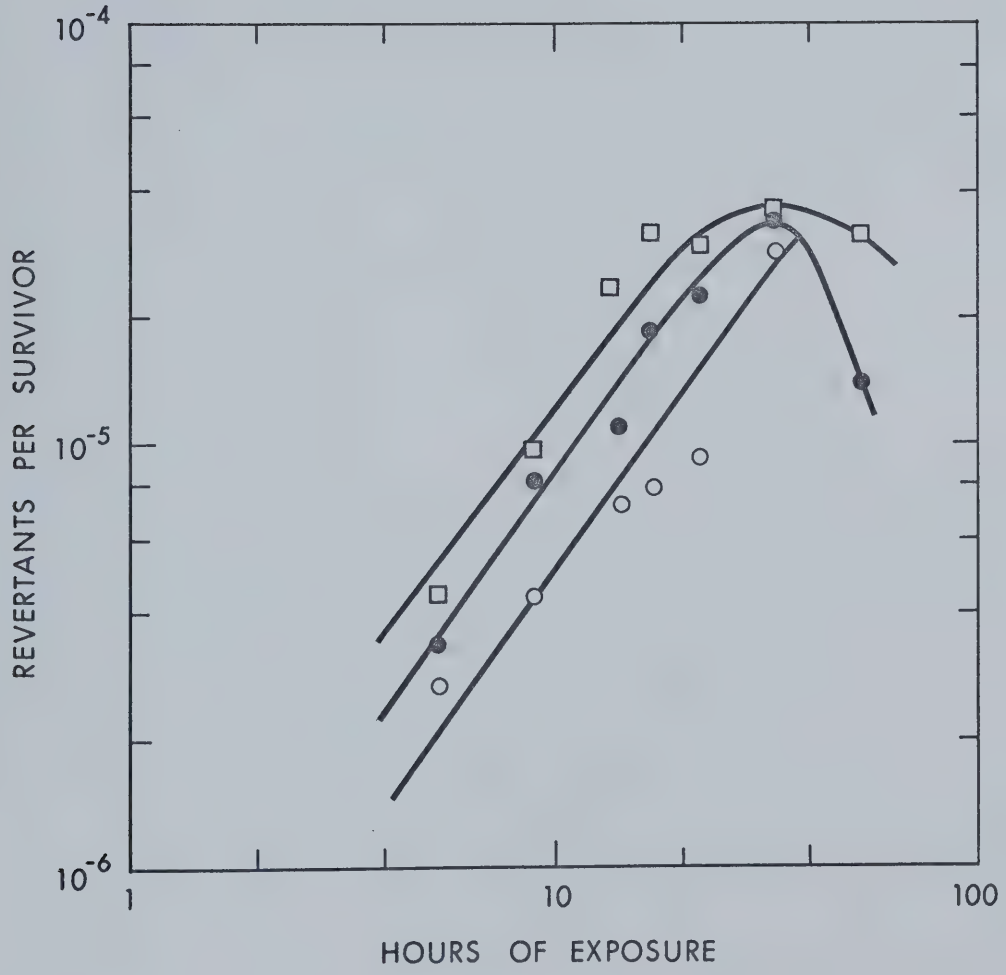

10^{-4}
10^{-5}
10^{-6}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

Figure 16. Reversion frequencies of *hom 3-10* after different hours of exposure to hycanthone, pH 7.0, under various lighting conditions.

○ hycanthone under dark conditions

● hycanthone under room lighting conditions

□ hycanthone under bright lighting conditions

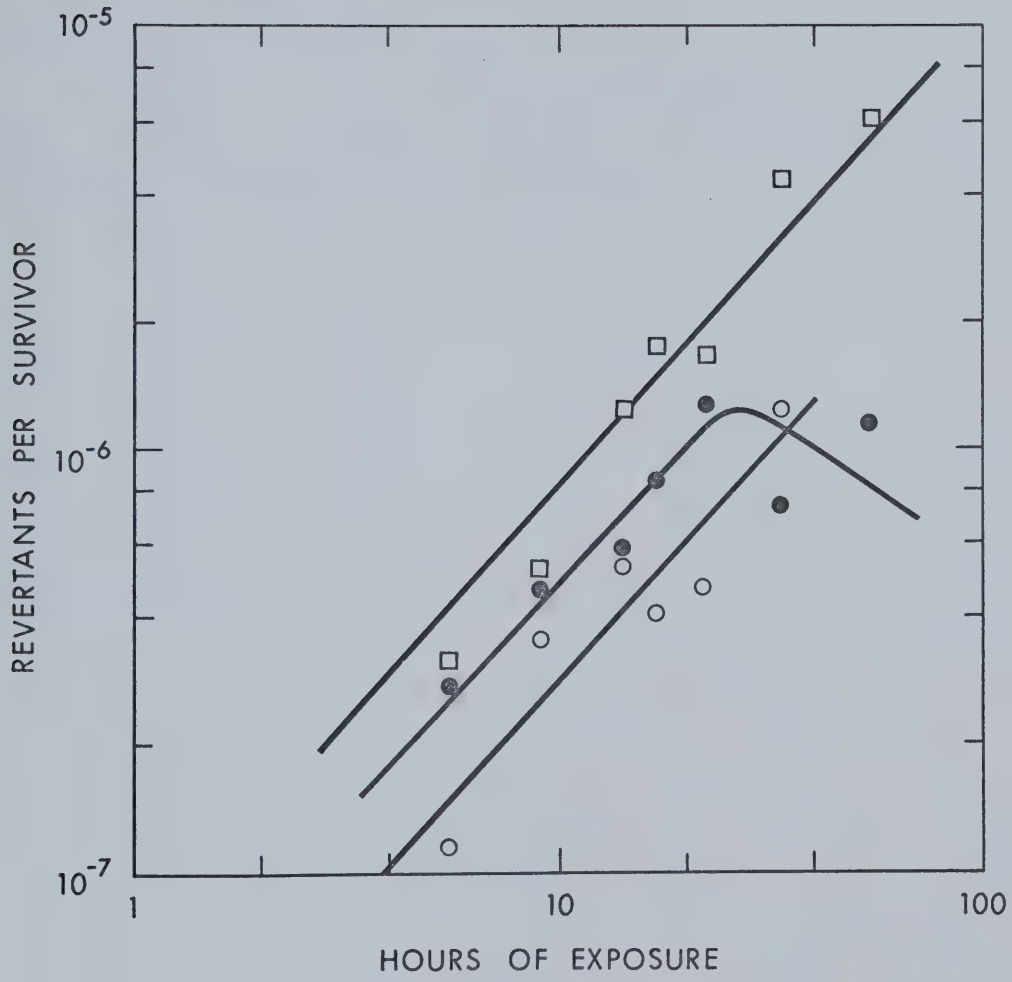

10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
1
10
100
HOURS OF EXPOSURE

TABLE 12. Lethality and mutagenicity of γ-irradiation on XV169-15A

Dose (Krad)	Survival (% of control)	Reversion Frequencies			
		his 1-7 ($\times 10^6$)	*hom 3-10* ($\times 10^7$)	*lys 1-1* total ($\times 10^6$)	*lys 1-1* locus ($\times 10^8$)
0	100	0	0	0	0
4.1	54.4	10.13	3.40	0.81	26.93
8.2	27.0	18.62	4.41	0.95	32.61
16.4	7.4	29.83	12.15	2.45	61.71
24.6	3.3	35.20	22.08	2.55	108.29
32.8	3.5	38.70	20.74	2.97	126.95

Figure 17. Survival of yeast after gamma-irradiation.

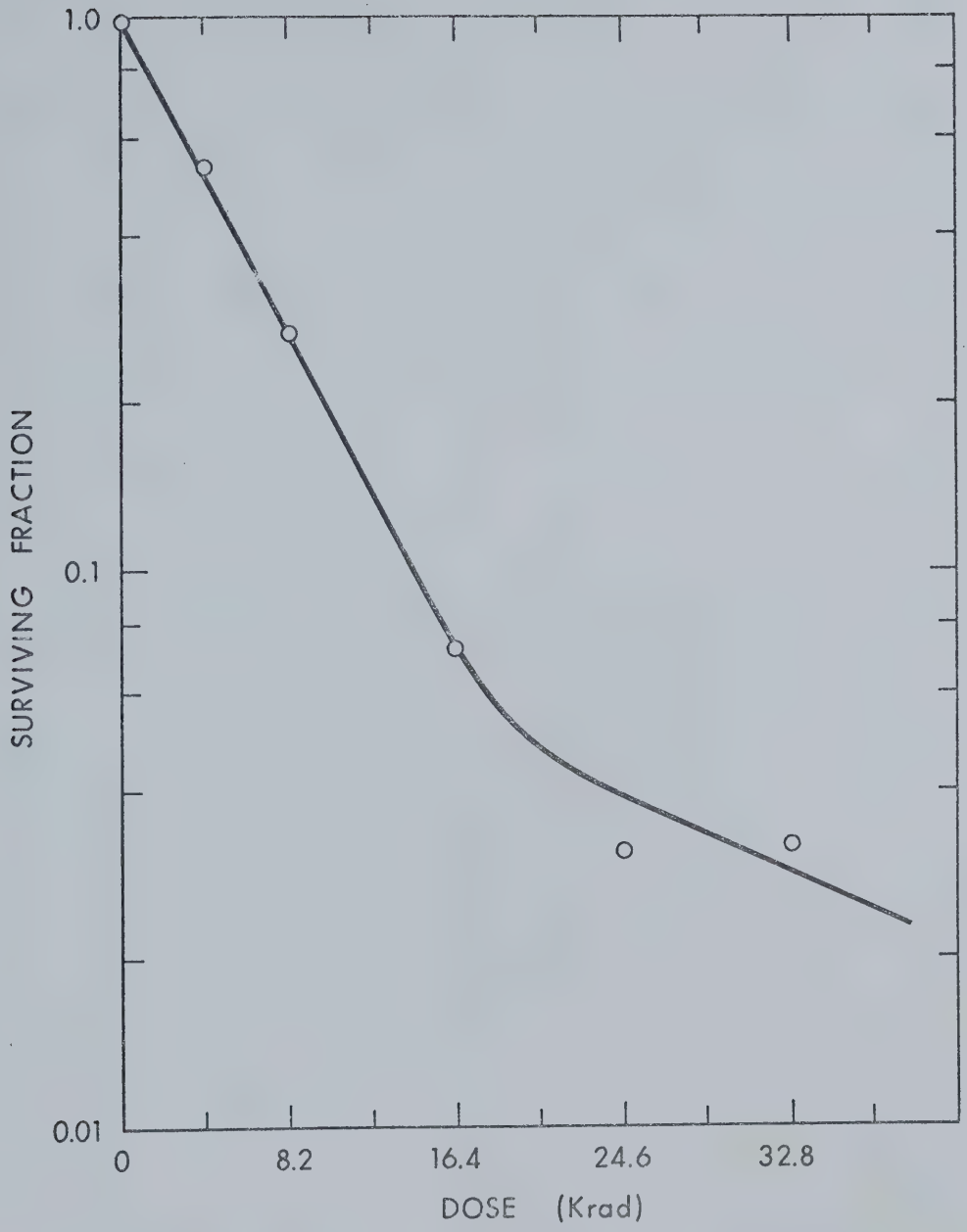
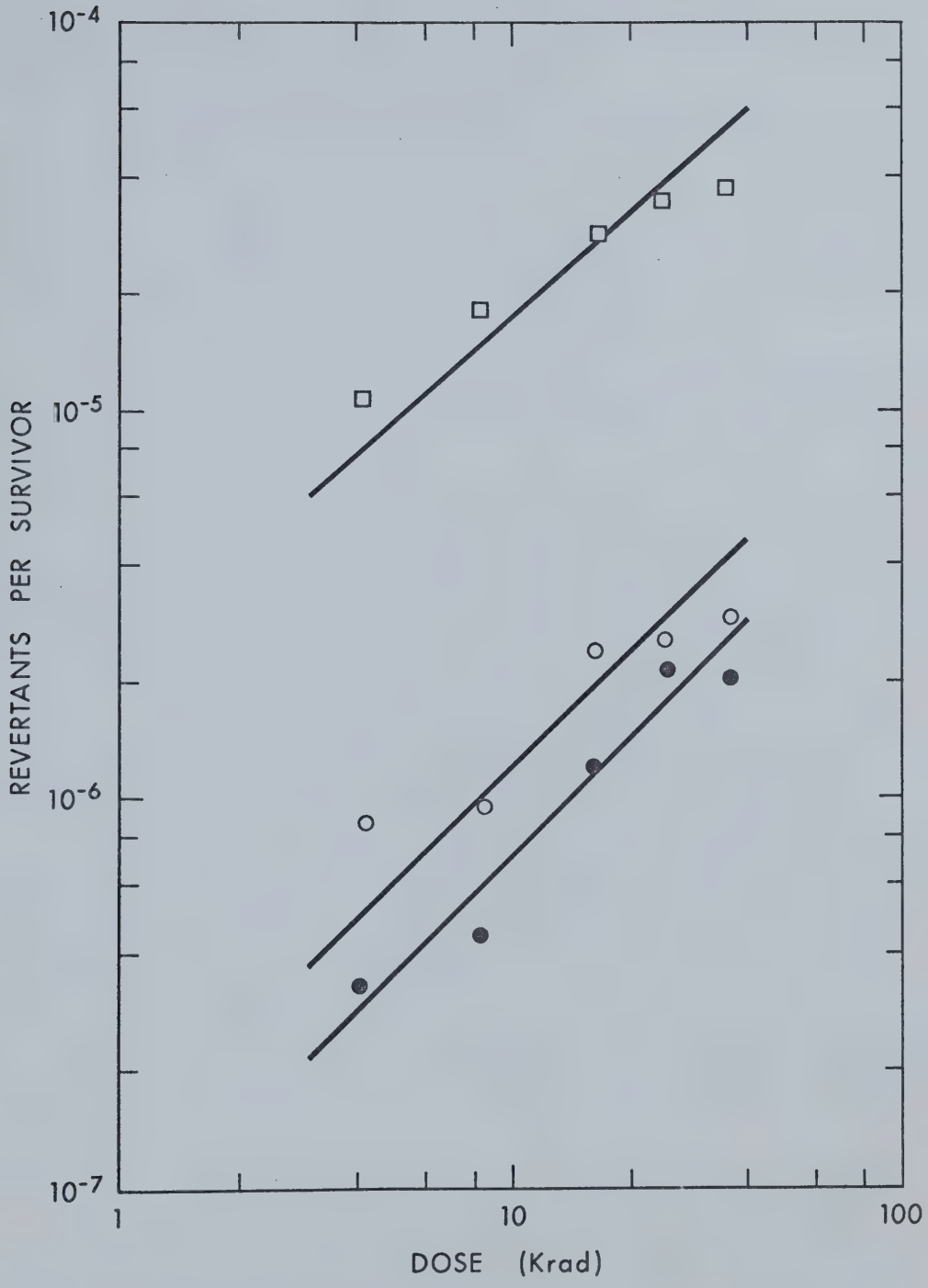

1.0
0.1
0.01
SURVIVING FRACTION
0
8.2
16.4
24.6
32.8
DOSE (Krad)

Figure 18. Reversion frequencies of *his 1-7*, *hom 3-10*, and *lys 1-1* after gamma-irradiation.

□ *his1-7*

○ *lys1-1*

● *hom3-10*


10^{-4}
10^{-5}
10^{-6}
10^{-7}
REVERTANTS PER SURVIVOR
1
10
100
DOSE (Krad)

The rates of reversions for all three markers are about the same, as is shown by the parallel nature of the slopes of the lines for the markers. The slope of each line is about one.

This differs from the action of hycanthone for the *hisI-7* locus, where a slope of two is observed.

E. Induction of Intragenic Recombination and Mitotic Crossing-Over by Hycanthone and Gamma-Rays in the Diploid X841

The strain of yeast, X841, was treated with varying concentrations of hycanthone at pH 7.0 for seven hours, and scored for survival and reversion to arginine independence.

The arginine independent cells can occur by two mechanisms: (1) intragenic recombination between the arg_{4-1} and the arg_{4-2} alleles, or (2) by reversion of the mutation at either of the mutant alleles. Both mechanisms are known to take place. Since the second event is rare compared to the frequency of the intragenic recombinational event, all arginine-independent colonies scored were taken to be recombinants.

Two experiments were done, treating the cells with hycanthone at the final concentrations shown in Table 13. The survival data and the recombination data for both experiments are presented also. Figures 19 and 20 show the graphs for this data.

For comparison, a gamma-ray experiment was done on the cells. The survival and recombination values are given in Table 14 and illustrated in Figures 21 and 22.

By comparison of the survival and the induction of prototrophs by gamma-rays and hycanthone, it appears that gamma-rays are much more efficient than hycanthone, i.e. gamma-rays kill less, but induce more

TABLE 13. Results of two experiments on survival, intragenic recombination and mitotic crossing-over as induced by various concentrations of hycanthone for seven hours of treatment

Experiment	Hycanthone (mg/ml)	Survival (% of control)	*arg 4* prototrophs ($\times 10^4$)	Frequency of red colonies and sectors ($\times 10^3$)
I	0	100	0	0
	0.031	96.5	0	1.00
	0.062	93.0	0.19	0.31
	0.125	96.5	0.65	1.00
	0.250	85.4	2.05	8.43
	0.50	84.0	2.86	8.64
	1.00	58.8	3.44	9.91
	2.00	32.0	3.49	8.76
II	0	100	0	0
	0.031	100	0	0.88
	0.062	100	0.37	2.64
	0.125	107.1	0.59	7.34
	0.250	94.0	1.78	6.62
	0.50	87.0	3.08	10.25
	1.00	72.1	3.35	15.01
	2.00	40.4	3.43	18.93

Figure 19. Survival of X841 after treatment with different concentrations of hycanthone, in two experiments.

● Experiment I

○ Experiment II

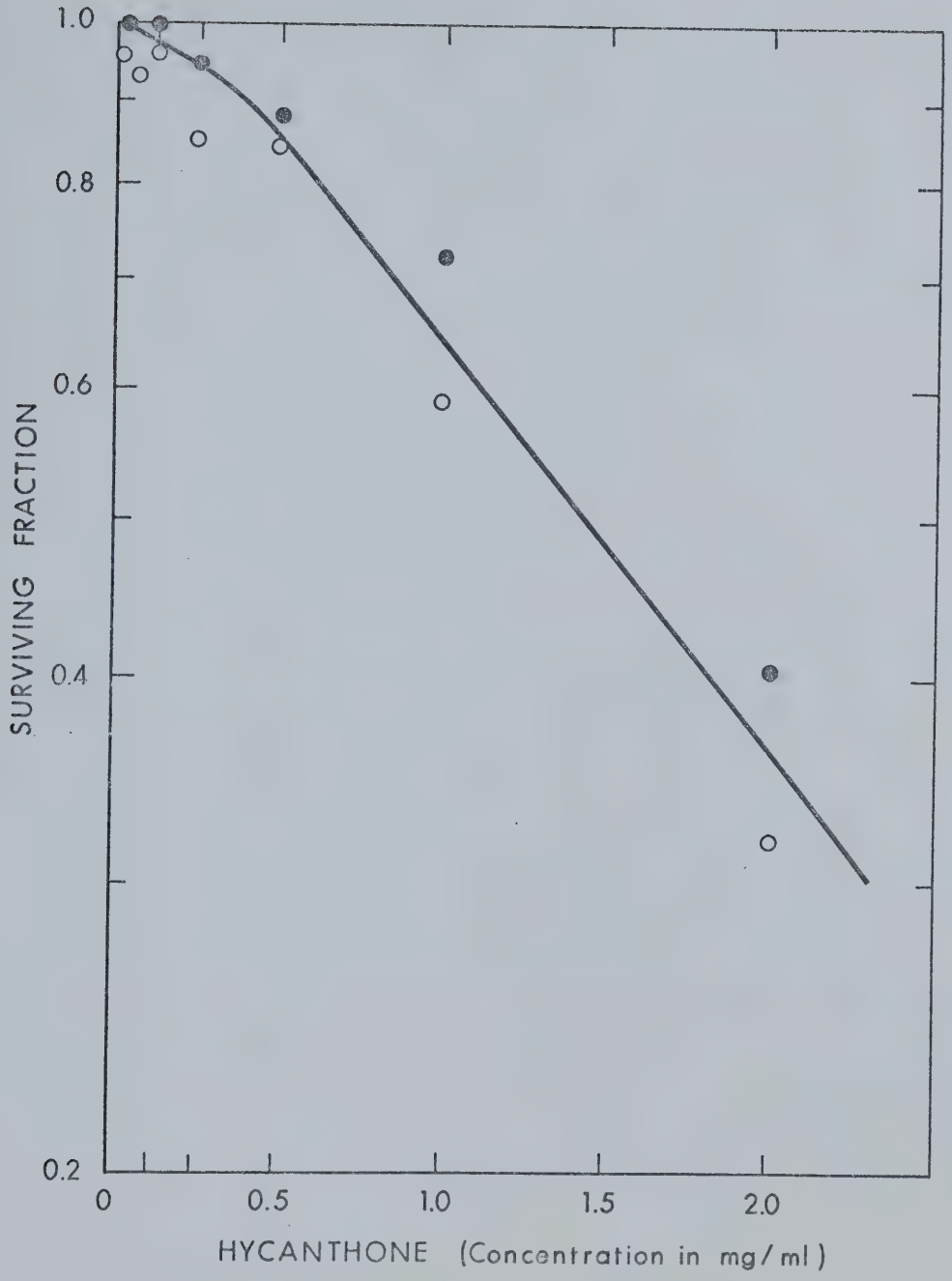

1.0
0.8
0.6
0.4
0.2
SURVIVING FRACTION
0
0.5
1.0
1.5
2.0
HYCANTHONE (Concentration in mg/ml)

Figure 20. Recombination frequencies of X841 after treatment with different concentrations of hycanthone, in two experiments.

● Experiment I

○ Experiment II

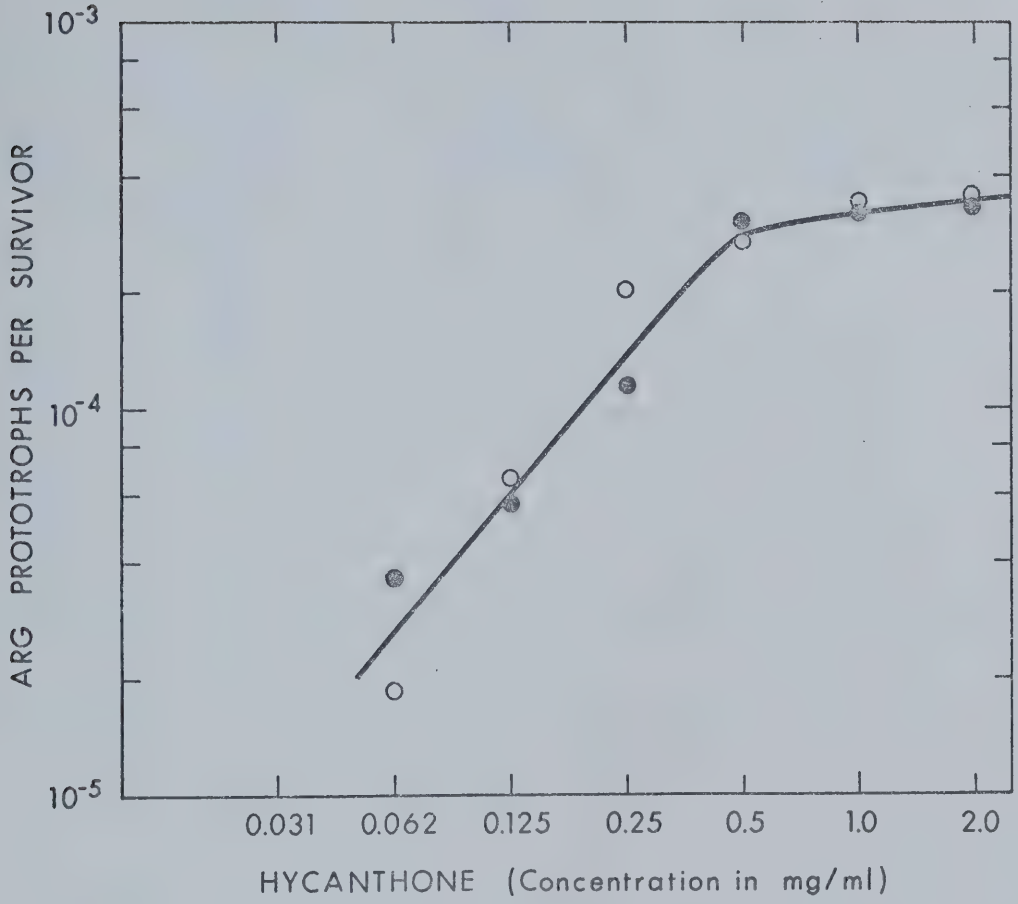

10^{-3}
10^{-4}
10^{-5}
ARG PROTOTROPHS PER SURVIVOR
0.031
0.062
0.125
0.25
0.5
1.0
2.0
HYCANTHONE (Concentration in mg/ml)

TABLE 14. Survival, intragenic recombination, and mitotic crossing-over as induced by γ-irradiation

Dose (Krad)	Survival (% of control)	*arg 4* prototrophs ($\times 10^4$)	Frequency of red colonies and sectors ($\times 10^3$)
0	100	0	0
4.1	99.6	2.42	15.41
8.2	91.6	3.55	21.86
16.4	68.6	6.62	32.73
24.6	52.9	7.85	48.69
32.8	39.6	11.37	45.95

Figure 21. Survival of X841 after gamma-irradiation.

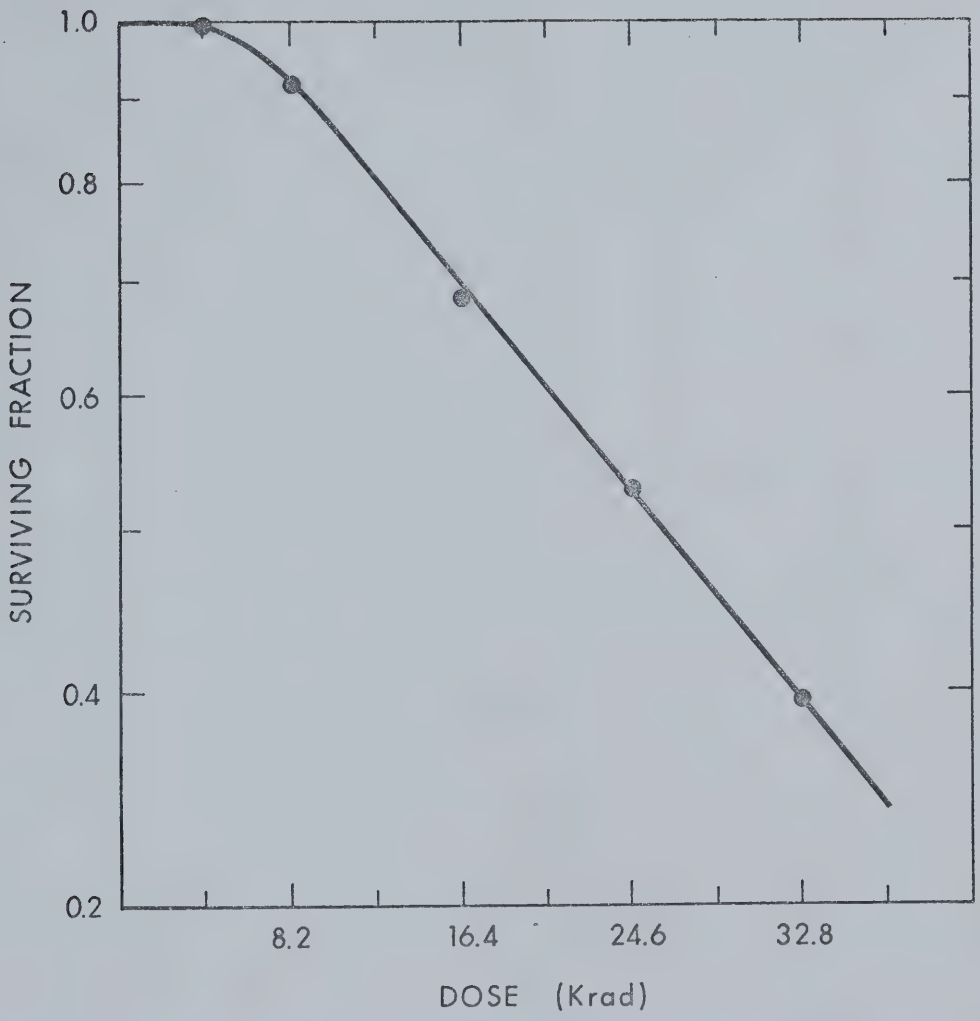
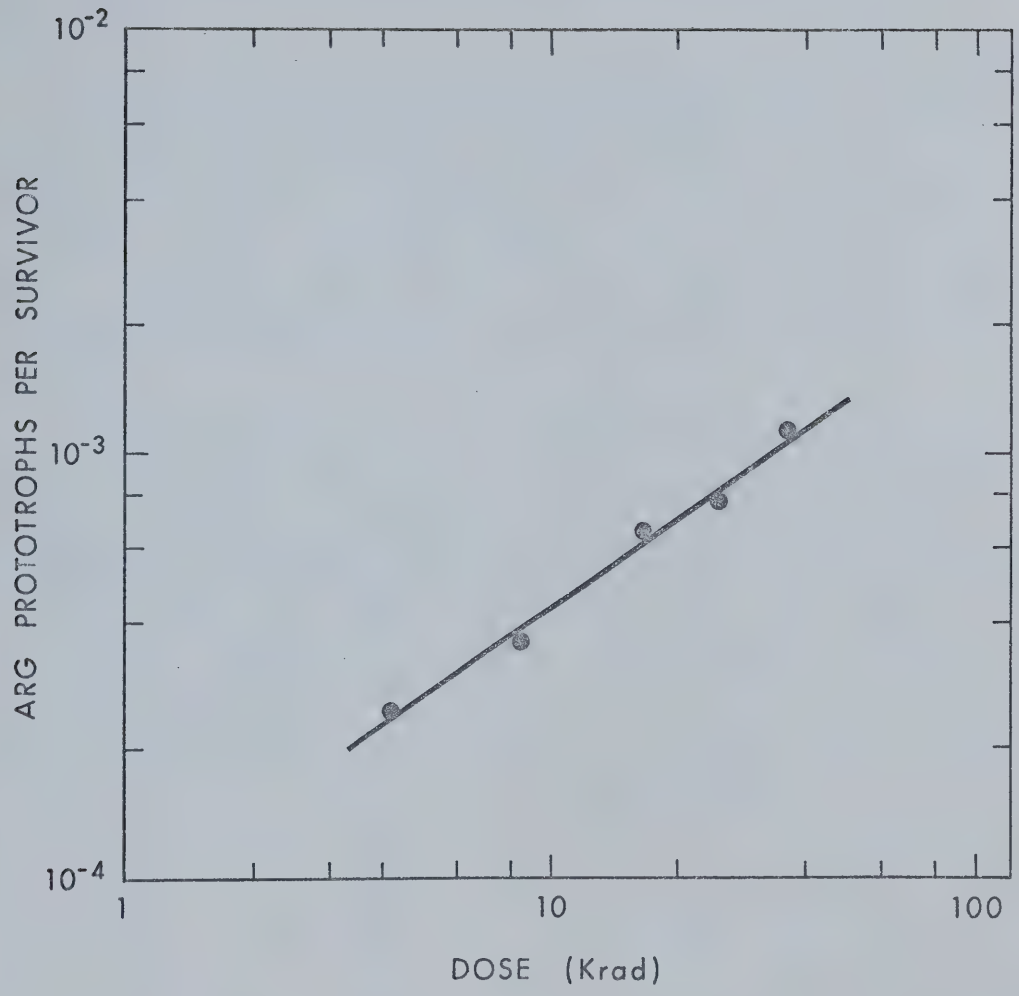

1.0
0.8
0.6
0.4
0.2
SURVIVING FRACTION
8.2
16.4
24.6
32.8
DOSE (Krad)

Figure 22. Recombination frequencies of X841 after gamma-irradiation.


10^{-2}
10^{-3}
10^{-4}
ARG PROTOTROPHS PER SURVIVOR
1
10
100
DOSE (Krad)

mutations. A mutation frequency of approximately 7.85×10^{-4} is obtained at 52.9 percent survival with gamma-rays, whereas a frequency of 3.4×10^{-4} is obtained at 58.8 percent survival with hycanthone treatment for seven hours.

The strain X841, being diploid, was suspected of being unstable; i.e. it could undergo sporulation if left in buffer or water. The cell suspension, once made, was used immediately and was not left overnight in the refrigerator as was done for the haploid strain. The cells were examined microscopically for the presence of asci before and after the experiment.

To test the genotype of the recombinants, a random sample of 52 arginine recombinants was picked from plates from each dose of either hycanthone or gamma-rays, and was replica-plated onto the following media: -his, -trp, -ade, -arg, -thr, YEPG, -met, -ura, -lys, -leu, and MC.

Those colonies that were arginine recombinants would have the following growth pattern:

Media	*Growth Pattern*
-his	-
-trp	-
YEPG	+
-arg	+
-thr	+
-met	+
-ura	+
-lys	+
-leu	+

Those that had formed spores might fail to grow on any of -ura, -lys,

-thr, -met, or -leu, or on any combination of these. Failure to grow on just one of them was taken as an indication that sporulation had occurred and that these were haploid cells.

Haploid colonies were found but there was not an increase in their numbers with dose or with time of treatment so that roughly the same number of haploid colonies occurred at each dose; the range of occurrence was from 2% to 10%. Therefore, it was concluded that sporulation was not induced by either hycanthone or by gamma-rays. The number of arginine recombinants due to meiosis was considered negligible.

A further interesting characteristic of the strain is the presence of the *ade2* gene, on fragment 1. If mitotic crossing-over were induced between the gene and the centromere, red colonies or sectored red and white colonies would appear on the YEPD plates. These aberrant colonies were counted and their frequency of occurrence calculated. This data is presented in the last column of Tables 13 and 14. With an increasing dose of either hycanthone or gamma-irradiation there is an increase in mitotic crossing-over.

DISCUSSION

The structure of hycanthone is a three planar one, similar to the structure of acridines. Brenner *et al.* (1961) postulated that acridines act as mutagens because they cause insertions or deletions of base pairs; i.e., acridines are bound to DNA by sliding between adjacent pairs, forcing them 6.8Å apart instead of 3.4Å. If this happens, they suggest that it can lead to the addition or the deletion of a base pair during replication.

Intercalation models for the binding of drugs to DNA require the uncoiling of the double helix. Such uncoiling in closed DNA results in, first, the removal and, with further intercalation, the reversal of the supercoils. These can be detected by changes in the sedimentation coefficient of the circular DNA (Waring, 1970*a*). Waring looked at the effects of 17 substances on the S_{20} of ϕX174 RF DNA. Using ethidium bromide as a standard, he showed that an equivalence between supercoils and accumulated drug-induced uncoiling of the helix occurred at a binding ratio, v_c, of 0.04 ± 0.008 ethidium bromide molecules bound per nucleotide.

Different values of v_c were found when different drugs were used, including hycanthone. Based on an unwinding angle of 12° for ethidium bromide, which is the minimum uncoiling needed to accommodate a planar aromatic ring between the stacked base pairs, the unwinding angle was calculated for hycanthone and was found to be less than 12°. Waring (1970*b*) attributes this discrepancy to the fact that a certain proportion of the "bound" drug stays in a non-intercalative state, and that there is an equilibrium between the outside bound drug and the intercalated drug.

The structure of ICR-170 is 2-methoxy-6-chloro-9-{3-[ethyl-2-

chloroethyl] aminopropylamino) acridine dihydrochloride; i.e., it is an acridine nucleus plus a monofunctional nitrogen mustard (Brockman, 1964). It has a dual action in that it causes both frameshift mutations and base changes (Auerbach and Kilbey, 1971). From Waring's evidence and from the fact that hycanthone has a structure similar to ICR-170, it could be expected that hycanthone would cause both frameshift mutations and base substitutions. Within the boundary conditions of the test system that has been used, it has been shown that this is the case.

When hycanthone is used at pH 7.0 and pH 5.9, it is found that the difference in pH does not affect the rate of the production of mutations; however, the overall reversion frequencies for all markers are about two times higher at pH 7.0 than at pH 5.9. Two possible explanations are: 1) that hycanthone is very sensitive to acid (Rossi *et al.*, 1965), such that it is slightly inactivated at the lower pH and is, therefore, less efficient, or 2) that the yeast cells are less receptive to the drug at the lower pH.

Hycanthone is more mutagenic when treatment of the cells is carried out in bright lighting conditions than in the dark. The enhancement is noticeable even in room lighting. A more pronounced photodynamic effect is seen for *hom3-10*, a reputed frameshift mutant.

Two types of dose-action studies were done with hycanthone; one was at fixed concentration of 0.125 mg/ml of hycanthone over various ranges of times of exposure; the other was at a fixed time of exposure to hycanthone which was administered at different concentrations. To compare these results, a new parameter was defined. It was called the "effective dose," and was calculated by multiplying the time of exposure

by the concentration of hycanthone used. The survival fraction of yeast is plotted against ED and is shown in Figure 23. For the sake of comparison, the data from 12 hours of exposure and from 8 hours of exposure were pooled here, as well as in Figure 27. As can be seen, longer exposure to low dose of hycanthone is more toxic to the yeast than the shorter, fixed time of exposure at increasing concentrations.

Figures 24, 25, and 26 show, respectively, the reversion frequencies for *his1-7*, *hom3-10*, and *lys1-1*, plotted against ED. From these, it can be seen that for mutation induction, again a longer exposure to low dose of hycanthone is more effective than shorter fixed exposure to increasing concentrations of the drug. It is worth pointing out that the production of mutations is directly correlated with lethality. The effect of non-reciprocity of time and concentrations has been observed before (Něcásek, 1966), but an explanation for this phenomenon has not been presented as yet. From these data, it appears that a longer exposure time is necessary for the effective action of hycanthone; whether this is due to the drug itself or to the physiology of the yeast cells is not known.

In order to draw some conclusions about the relative mutagenicity of hycanthone in the two types of dose-action studies, and gamma irradiation, the reversion frequencies of *his1-7*, *hom3-10*, and *lys1-1* were plotted against survival.

Figure 27 shows the reversion frequencies for the three markers, in four experiments with hycanthone at pH 7.0, where concentration was fixed and the time of exposure was varied. Figure 28 shows the reversion frequencies for the three markers, after treatment with hycanthone for a

Figure 23. Survival plotted against Effective Dose of hycanthone obtained from four experiments.

● long exposure time at 0.125 mg/ml of hycanthone

□ 8 hours of exposure to increasing concentrations of hycanthone

△ 12 hours of exposure to increasing concentrations of hycanthone

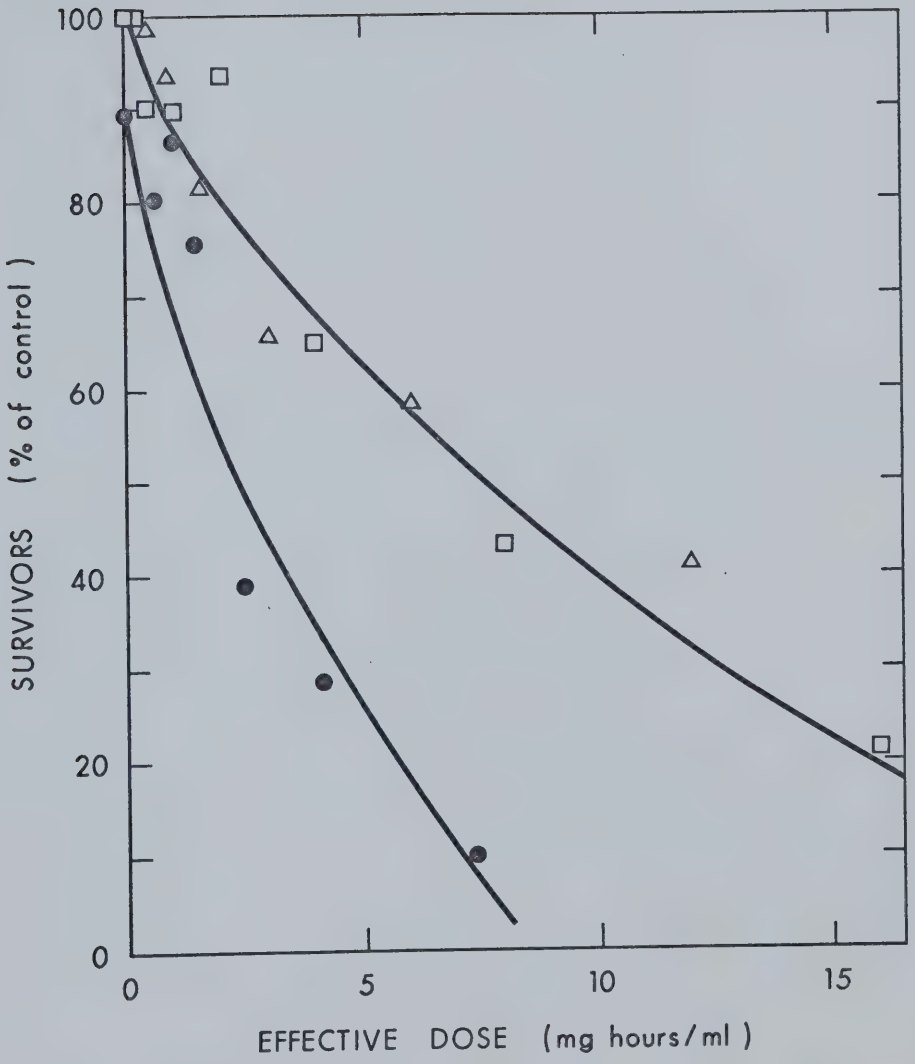

100
80
60
40
20
0
SURVIVORS (% of control)
0
5
10
15
EFFECTIVE DOSE (mg hours/ml)

Figure 24. Reversion frequencies obtained from *his1-7* studies plotted against Effective Dose of hycanthone.

○ after long exposure time to fixed concentration

△ 12 hours of exposure, concentration varied

● 8 hours of exposure, concentration varied

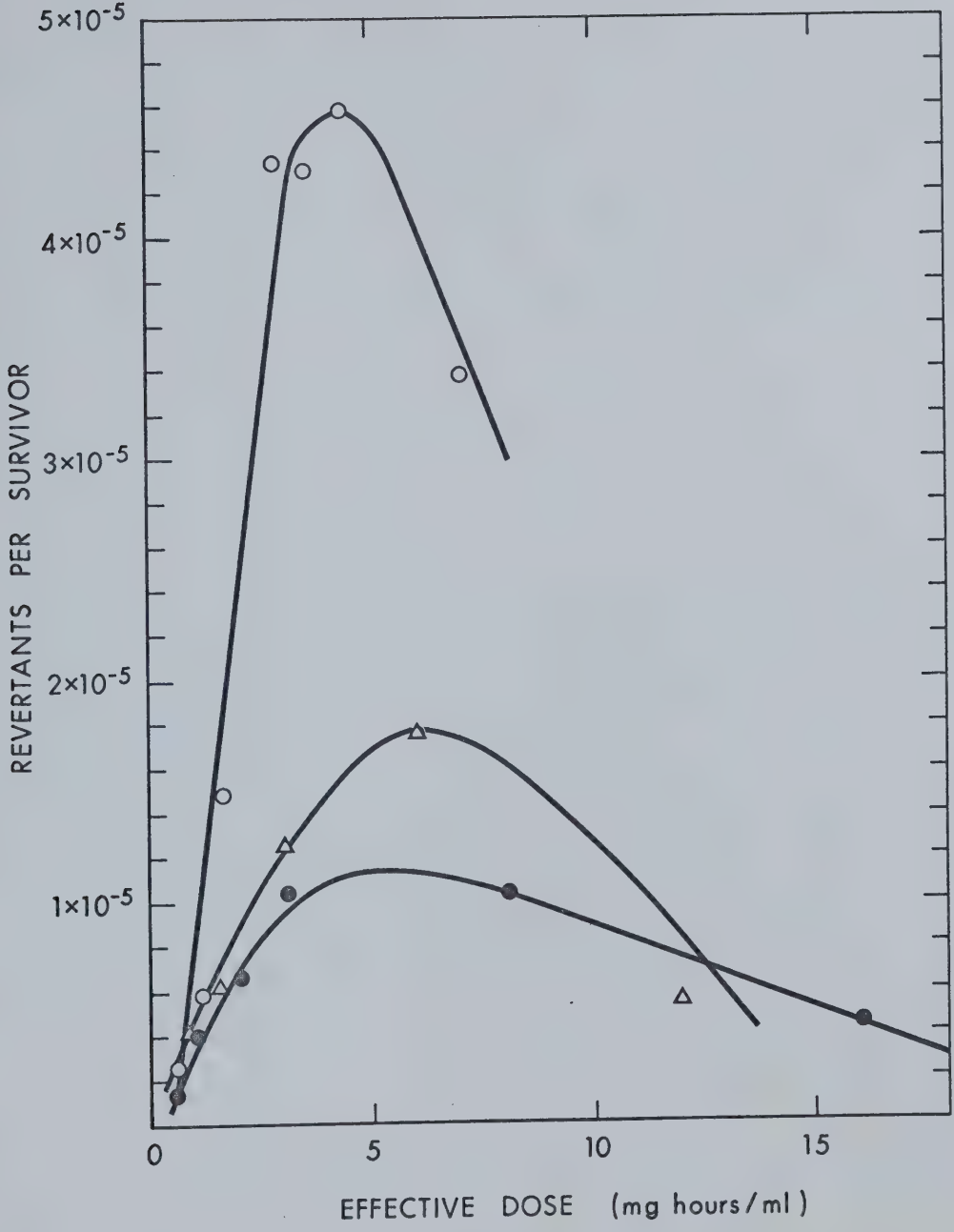

5×10⁻⁵
4×10⁻⁵
3×10⁻⁵
2×10⁻⁵
1×10⁻⁵
REVERTANTS PER SURVIVOR
0
5
10
15
EFFECTIVE DOSE (mg hours/ml)

Figure 25. Reversion frequencies obtained from *hom3-10* studies plotted against Effective Dose of hycanthone.

○ after long exposure time to fixed concentration

△ 12 hours of exposure, concentration varied

● 8 hours of exposure, concentration varied

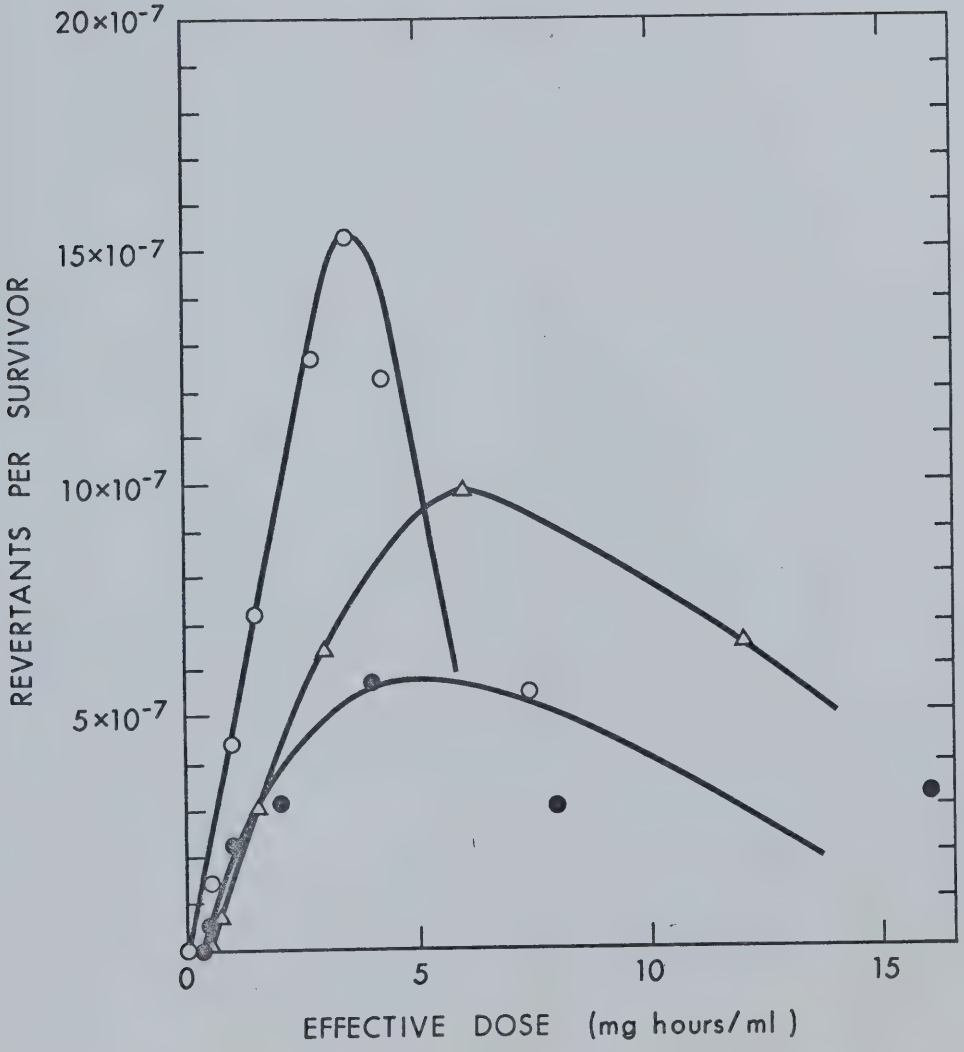

20×10⁻⁷
15×10⁻⁷
10×10⁻⁷
5×10⁻⁷
REVERTANTS PER SURVIVOR
0
5
10
15
EFFECTIVE DOSE (mg hours/ml)

Figure 26. Reversion frequencies obtained from *lys1-1* studies plotted against Effective Dose of hycanthone.

○ after long exposure time to fixed concentration

△ 12 hours of exposure, concentration varied

● 8 hours of exposure, concentration varied

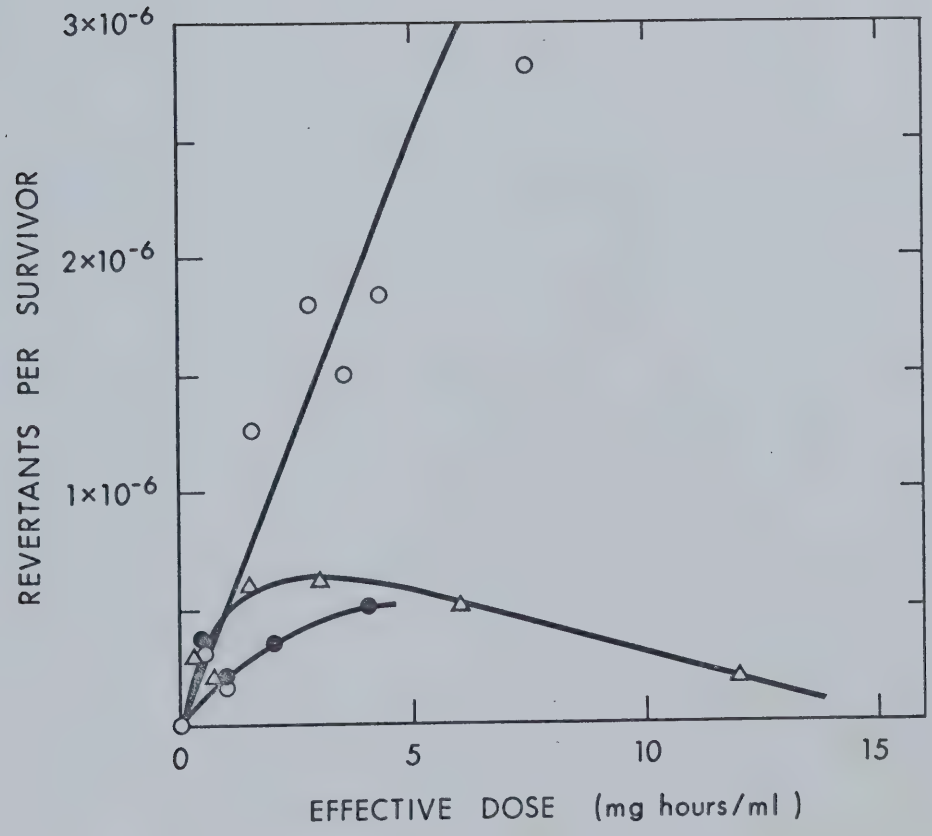

3×10⁻⁶
2×10⁻⁶
1×10⁻⁶
REVERTANTS PER SURVIVOR
0
5
10
15
EFFECTIVE DOSE (mg hours/ml)

Figure 27. Reversion frequencies plotted against survival: fixed concentration of hycanthone with time of exposure variable.

● *his1-7*

□ *hom3-10*

▲ *lys1-1*

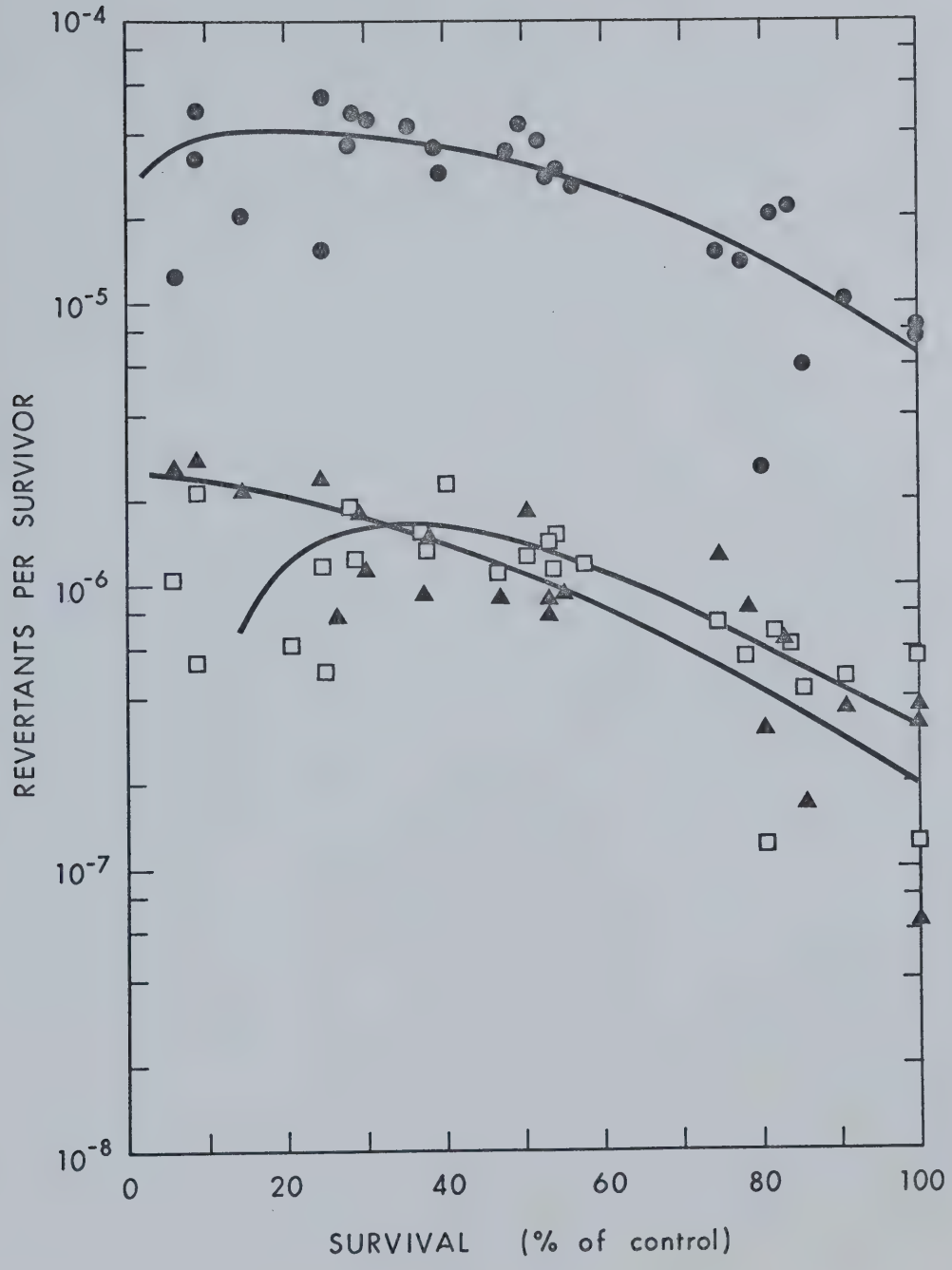
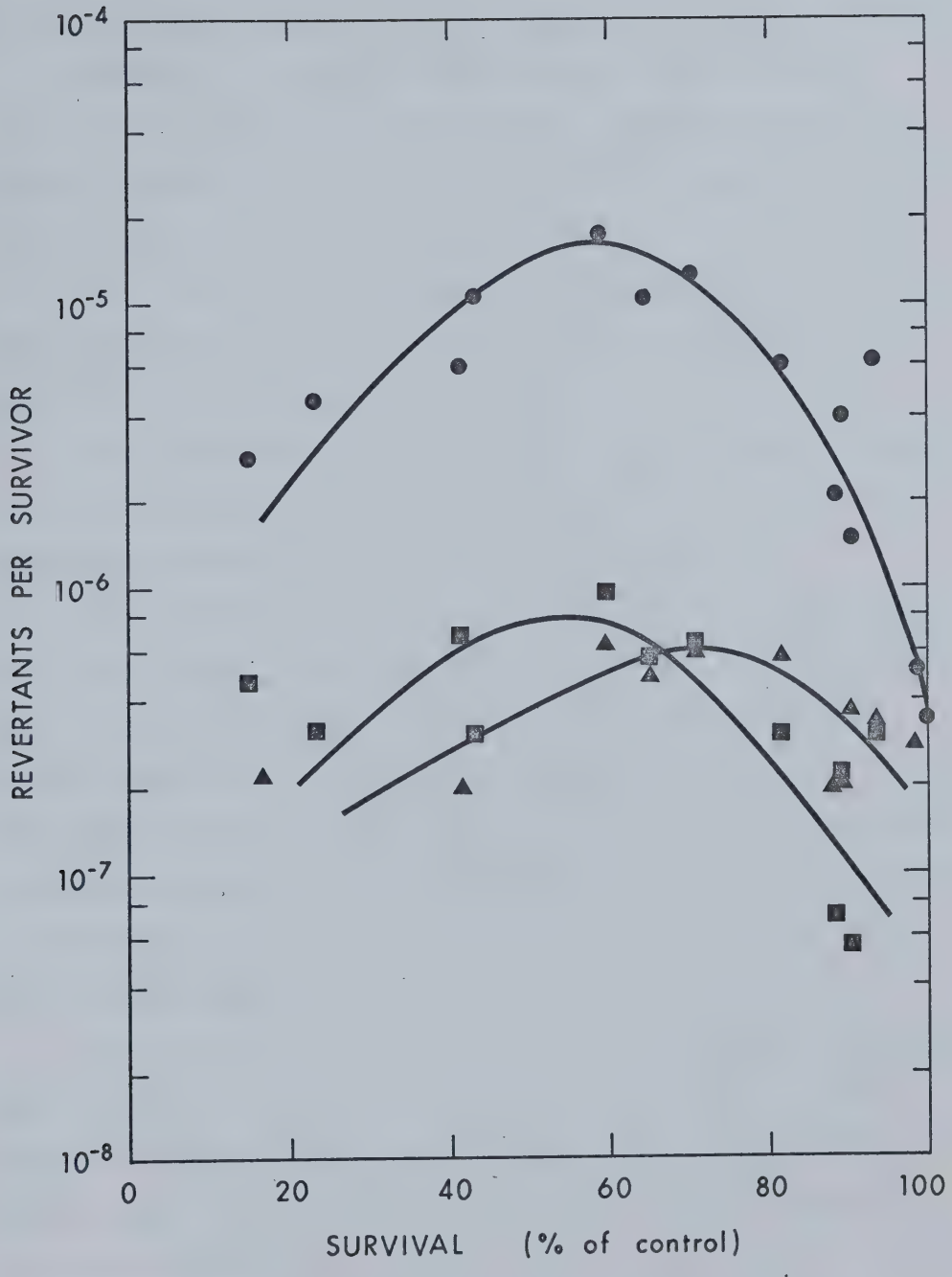

10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
0
20
40
60
80
100
SURVIVAL (% of control)

Figure 28. Reversion frequencies plotted against survival: fixed time of exposure with concentration of hycanthone varied.

● *his1-7*

■ *hom3-10*

▲ *lys1-1*


10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
0
20
40
60
80
100
SURVIVAL (% of control)

fixed time, but with varying concentrations, at pH 7.0. Figure 29 shows the gamma-radiation-induced reversion frequencies for the three markers.

Arbitrarily, the reversion frequencies of each marker were compared at 50% survival. At 50% survival, at different times of exposure to a constant concentration of hycanthone, the reversion frequencies of the markers are: *his1-7*, 3.1×10^{-5}; *hom3-10*, 1.4×10^{-6}; *lys1-1*, 1.1×10^{-6}. When the time of exposure is constant, but the drug concentration is varied, at 50% survival the reversion frequency of *his1-7* is 1.5×10^{-5}; of *hom3-10* is 0.76×10^{-6}; and of *lys1-1* is 0.37×10^{-6}.

After exposure to gamma-rays, the 50% survival reversion frequencies are *his1-7*, 1.3×10^{-5}; *hom3-10*, 1.5×10^{-6}; and *lys1-1*, 0.38×10^{-6}. Provisional conclusions can be drawn from these frequencies.

First, the relative mutation spectrum of the three markers is the same, whether time or concentration is varied, in the hycanthone experiments. Second, after a long exposure to a fixed concentration, the reversion frequency for each marker is approximately twice that obtained after fixed exposure to increasing concentrations. Third, qualitatively the mutation spectrum of the three markers is the same for gamma-rays and for hycanthone. Fourth, quantitatively, hycanthone in general appears to be as efficient as gamma-rays for induction of mutations.

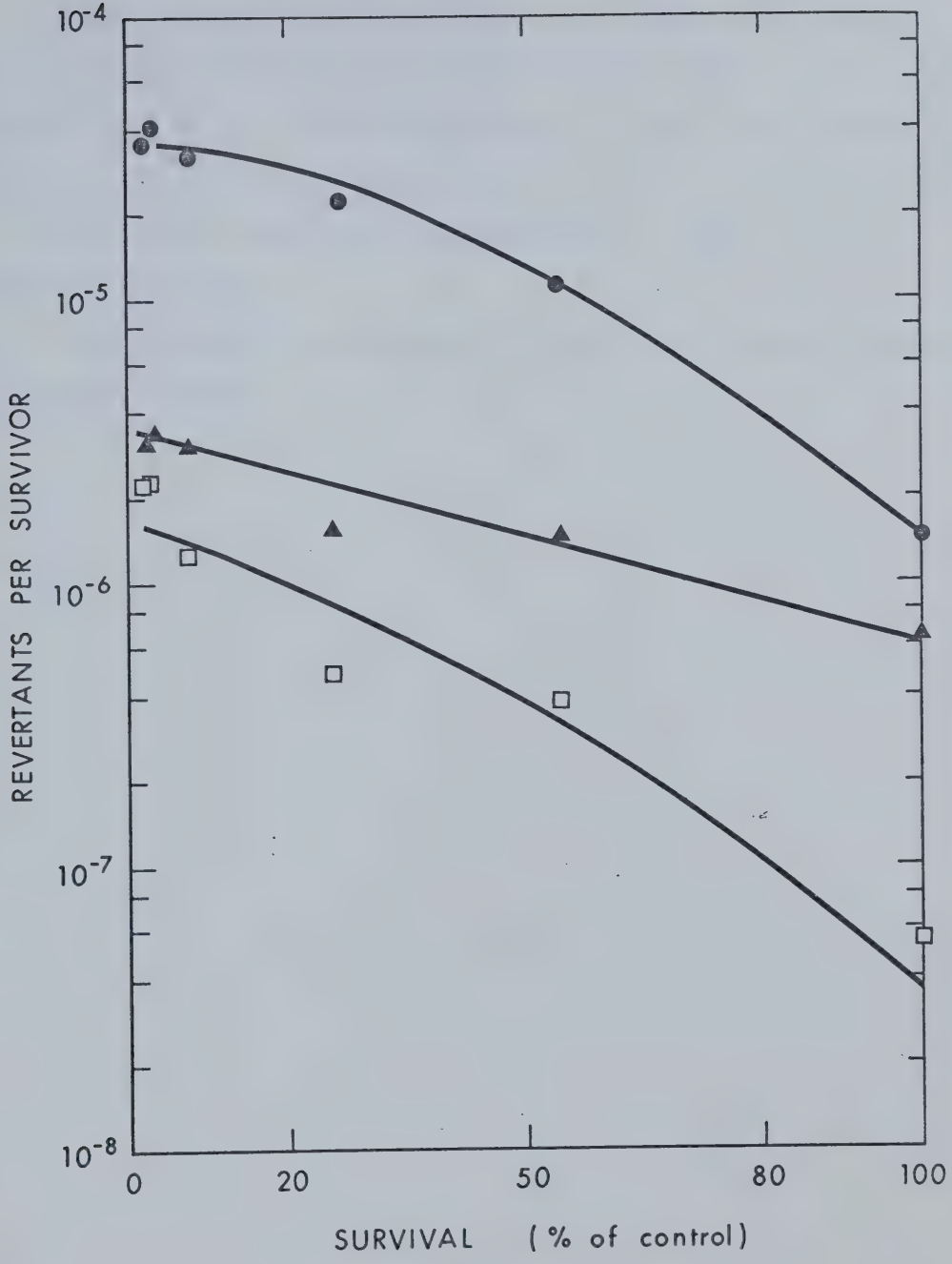
The results from the haploid strain of yeast XV169-15A show that hycanthone induces the reversions of the *his1-7*, *hom3-10*, and *lys1-1* markers, which are believed to be a missense mutation, a frameshift mutation, and a nonsense mutation, respectively. The experiments with the diploid X841 show that hycanthone induces crossing-over and intragenic recombination, both of which would indicate chromosome breakage.

Figure 29. Reversion frequencies plotted against survival: gamma-radiation.

● *his1-7*

□ *hom3-10*

▲ *lys1-1*


10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
REVERTANTS PER SURVIVOR
0
20
50
80
100
SURVIVAL (% of control)

On the contrary, IA-4 was not found to be mutagenic in the system used, at pH 5.9. There could be any number of explanations for this. The fact that IA-4 is toxic at a concentration of 0.5 mg/ml, but not mutagenic, perhaps could indicate that the compound is acting at the cell surface to kill the cell but is not being taken into the cell so that it is not reacting with the DNA.

In conclusion, hycanthone acts on the yeast *Saccharomyces cerevisiae* as a general mutagen.

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APPENDIX

TABLE A1 (a). Survival of yeast after different hours of exposure to hycanthone and IA-4

Treatment Time (Hours)	Colony Counts					
	H_2O	Buffer pH 5.9	Buffer pH 7.0	IA-4 pH 5.9	Hycanthone pH 5.9	Hycanthone pH 7.0
0	1257	1111	1226	1021	1238	1130
4.5	1216	1205	1086	--	1095	1008
8	1046	1020	1109	1005	1054	1090
12.5	1146	1233	1072	1071	1011	947
22	1196	1108	1152	874	899	636
28	1248	1101	1204	--	1170	472
34	1073	1146	1119	851	856	367
59	1145	1078	1029	756	482	125

TABLE A1 (b) Colony counts for revertants after different hours of exposure to water, phosphate buffers, pH 5.9 and pH 7.0, hycanthone at pH 5.9 and pH 7.0, and IA-4 at pH 5.9

Condition	Treatment Time (hrs)	Colony Counts				locus
		YEPD	-his	-thr	-lys	
Water	0	1257	210	10	84	5
	4.5	1216	206	3	65	1
	8	1046	176	5	72	4
	12.5	1146	190	6	56	2
	22	1196	206	6	75	1
	28	1248	228	12	77	3
	34	1073	206	5	90	1
	59	1145	178	7	84	0
Buffer at pH 5.9	0	1111	224	7	72	0
	4.5	1205	226	10	68	2
	8	1020	160	4	71	2
	12.5	1233	166	6	73	1
	22	1108	222	8	62	1
	28	1101	196	5	66	2
	34	1146	200	7	77	1
	59	1078	184	3	75	1
Buffer at pH 7.0	0	1226	254	1	74	0
	4.5	1087	206	2	66	1
	8	1109	224	7	86	3
	12.5	1072	202	7	47	1
	22	1152	224	3	68	3
	28	1204	205	8	98	1
	34	1119	200	7	75	1
	59	1029	192	9	72	1

TABLE A1 (b) (Cont'd)

Condition	Treatment Time (hrs)	Colony Counts YEPD	-his	-thr	-lys	locus
IA-4 at pH 5.9	0	1021	190	0	35	1
	45	--	--	--	--	-
	8	1005	104	2	13	1
	12.5	1071	160	4	21	1
	22	874	122	3	18	1
	28	--	--	--	--	-
	34	851	116	6	14	2
	59	756	124	10	7	0
Hycanthone pH 5.9	0	1238	218	4	86	1
	45	1094	260	9	78	4
	8	1054	382	18	78	2
	12.5	1011	650	29	104	2
	22	899	2210*	48	211	6
	28	1170	2830*	68	313	9
	34	856	3460*	63	281	11
	59	482	3050*	62	271	12
Hycanthone pH 7.0	0	1130	194	1	73	1
	4.5	1008	454	16	93	1
	8	1090	884	54	104	4
	12.5	947	1630	75	157	6
	22	636	2890*	81	151	-
	28	472	2110*	77	110	4
	34	367	1770*	47	93	4
	59	125	448	8	44	1

*counted at 10^{-4} dilution

TABLE A1 (c) Reversion frequencies (x 10^6) for *his1-7* revertants induced by hycanthone and IA-4 after different hours of exposure

Treatment Time (Hours)	H_2O	Buffer pH 5.9	Buffer pH 7.0	IA-4 pH 5.9	Hycanthone pH 5.9	Hycanthone pH 7.0
0	1.67	2.02	2.07	1.86	1.76	1.70
4.5	1.69	1.88	1.89	--	2.37	4.50
8	1.68	1.57	2.02	1.04	3.62	8.11
12.5	1.66	1.35	1.88	1.49	6.43	17.2
22	1.72	2.00	1.94	1.39	24.6	45.4
28	1.83	1.78	1.70	--	24.2	44.7
34	1.92	1.75	1.79	1.36	40.4	48.2
59	1.56	1.71	1.87	1.64	63.3	35.8

TABLE A1 (d) Reversion frequencies (x 10^7) for *hom3-10* revertants induced by hycanthone and IA-4 after different hours of exposure

Treatment Time (Hours)	H_2O	Buffer pH 5.9	Buffer pH 7.0	IA-4 pH 5.9	Hycanthone pH 5.9	Hycanthone pH 7.0
0	0.80	0.63	0.08	0	0.32	0.09
4.5	0.25	0.83	0.18	--	0.82	1.6
8	0.17	0.39	0.63	0.20	1.7	5.0
12.5	0.52	0.47	0.65	0.37	2.9	7.9
22	0.50	0.72	0.26	0.34	5.3	13
28	0.96	0.45	0.66	--	5.8	16
34	0.47	0.61	0.63	0.71	7.4	13
59	0.61	0.28	0.88	1.3	13	6.4

TABLE A1 (e) Reversion frequencies (x 10^6) for *lys1-1* revertants induced by hycanthone and IA-4 after different hours of exposure

Treatment Time (Hours)	H_2O	Buffer pH 5.9	Buffer pH 7.0	IA-4 pH 5.9	Hycanthone pH 5.9	Hycanthone pH 7.0
0	0.67	0.65	0.60	0.34	0.70	0.65
4.5	0.53	0.56	0.61	--	0.71	0.92
8	0.69	0.70	0.78	0.13	0.74	0.95
12.5	0.49	0.59	0.44	0.20	1.0	1.7
22	0.65	0.56	0.59	0.21	2.4	2.4
28	0.62	0.56	0.81	--	2.7	2.3
34	0.84	0.67	0.67	0.17	3.3	2.5
59	0.73	0.70	0.67	0.09	5.6	3.5

TABLE A1 (f) Locus reversion frequencies (x 10^8) for *lys1-1* revertants induced by hycanthone and IA-4 after different hours of exposure

Treatment Time (Hours)	H_2O	Buffer pH 5.9	Buffer pH 7.0	IA-4 pH 5.9	Hycanthone pH 5.9	Hycanthone pH 7.0
0	4	0	0	1	1	1
4.5	1	2	1	--	4	1
8	4	2	3	1	2	4
12.5	2	1	1	1	2	6
22	1	1	3	1	7	--
28	2	2	1	--	8	8
34	1	1	1	2	13	11
59	0	1	1	0	25	8

TABLE A3 (a) Colony counts for revertants, after different hours of exposure to buffer and hycanthone at pH 7.0

Condition	Treatment Time (hrs)	Colony Counts			
		YEPD	-his	-thr	-lys
Buffer pH 7.0	0	968	221	7	82
	7	1141	222	2	81
	18	857	222	8	88
	24	1020	228	5	60
	27	984	218	6	81
	30	1013	150	5	59
	33	1065	194	5	75
	55	938	14	4	78
Hycanthone pH 7.0	0	1047	237	4	81
	7	972	952	52	104
	18	522	2070*	87	99
	24	467	1730*	55	79
	27	376	1400*	52	65
	30	302	1400*	43	52
	33	247	1390*	29	77
	55	92	540	16	34

*Counted at a dilution of 10^{-1}

TABLE A3 (Cont'd)

(b) Reversion frequencies (x 10^6) for *hisI-7* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	pH 7.0	hycanthone	-control
0	2.28	2.26	--
7	1.95	9.79	7.84
18	2.59	39.73	37.14
24	2.24	37.05	34.81
27	2.22	37.23	35.01
30	1.48	46.36	44.88
33	1.82	56.28	54.46
55	0.149	58.70	58.55

(c) Reversion frequencies (x 10^6) for *hom3-I0* revertants induced by hycanthone at pH 7.0 after different hours of exposure

0	0.072	0.038	--
7	0.017	0.535	0.518
18	0.093	1.667	1.574
24	0.049	1.178	1.129
27	0.061	1.383	1.322
30	0.050	1.424	1.374
33	0.047	1.174	1.127
55	0.043	1.739	1.696

TABLE A3 (Cont'd)

(d) Reversion frequencies (x 10^6) for *lysI-1* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	pH 7.0	hycanthone	-control
0	0.847	.774	--
7	0.710	1.070	.360
18	1.027	1.897	.870
24	0.588	1.692	.904
27	0.823	1.729	.906
30	0.582	1.722	1.140
33	0.704	3.117	2.413
55	0.832	3.696	2.864

(e) Colony counts for revertants after different hours of exposure to buffer and hycanthone at pH 7.0

Condition	Treatment Time (hrs)	Colony Counts				
		YEDP	-his	-thr	-lys	locus
Buffer pH 7.0	0	867	99	2	241	2
	6.5	925	85	4	253	3
	16.5	1028	91	1	234	3
	18	943	83	8	207	0
	20	998	77	0	234	2
	23	918	95	4	256	0
	25.5	927	75	3	270	0
	30.5	967	106	3	247	2
	51	963	94	2	243	1

TABLE A3 (e) (Cont'd)

Condition	Treatment Time (hrs)	Colony Counts YEDP	-his	-thr	-lys	locus
Hycanthone pH 7.0	0	844	104	4	231	0
	6.5	895	247	15	253	1
	16.5	786	888	38	209	7
	18	675	1047	44	207	5
	20	712	1066	46	166	7
	23	497	1306	61	128	3
	25.5	353	1064	81	80	2
	30.5	244	908	46	56	5
	51	56	74	6	21	0

(f) Reversion frequencies (x 10^6) for *his1-7* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	1.142	1.177	0.035
6.5	0.919	2.780	1.861
16.5	0.885	11.298	10.413
18	0.880	15.511	14.831
20	0.772	21.629	20.857
23	1.035	26.278	25.243
25.5	0.809	30.142	29.333
30.5	1.096	37.213	36.117
51	0.976	13.214	12.238

TABLE A3 (Cont'd)

(g) Reversion frequencies (x 10^6) for *hom3-10* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	.0231	.0423	.0192
6.5	.0432	.1676	.1244
16.5	.0097	.4835	.4738
18	.0848	.6519	.5671
20	0	.6461	.6461
23	.0436	1.2274	1.1838
25.5	.0324	2.2946	2.2622
30.5	.0310	1.8853	1.8543
51	.0207	1.0714	1.0507

(h) Reversion frequencies (x 10^6) for *lys1-1* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	2.779	2.6131	--
6.5	2.735	2.8268	.092
16.5	2.276	2.6590	.383
18	2.195	3.0667	.862
20	2.345	2.3315	--
23	2.789	2.5755	--
25.5	2.913	2.2663	--
30.5	2.554	2.2951	--
51	2.513	3.750	--

TABLE A3 (Cont'd)

(i) Colony counts for revertants after different hours of exposure to buffer and hycanthone at pH 7.0

Condition	Treatment time (hrs)	Colony Counts YEDP	-his	-thr	-lys	locus
Buffer pH 7.0	0	1052	155	2	85	1
	12	1121	153	1	87	3
	18	1159	141	2	96	1
	22	1313	139	3	86	1
	24	1150	142	5	98	1
	48	1252	143	9	100	1
	66	1231	150	2	91	1
Hycanthone pH 7.0	0	1096	151	6	95	2
	12	1054	967	63	125	6
	18	887	2040	57	131	9
	22	564	1640	81	84	7
	24	573	1760	66	92	6
	48	262	428	16	42	2
	66	155	345	10	47	5

TABLE A3 (Cont'd)

(j) Reversion frequency (x 10^6) for *his1-7* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	1.473	1.378	--
12	1.365	9.175	7.810
18	1.217	22.999	21.782
22	1.059	29.078	28.018
24	1.235	30.716	29.481
48	1.142	16.336	15.194
66	1.219	22.258	21.039

(k) Reversion frequency (x 10^6) for *hom3-10* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	.0190	.0547	.0357
12	.0039	.5977	.5888
18	.0173	.6426	.6253
22	.0229	1.4362	1.4133
24	.0435	1.1518	1.1083
48	.0719	.6107	.5388
66	.0163	.6452	.6289

(l) Reversion frequency (x 10^6) for *lys1-1* revertants induced by hycanthone at pH 7.0 after different hours of exposure

Treatment time (hrs)	Buffer pH 7.0	Hycanthone pH 7.0	-control
0	.801	.867	.066
12	.776	1.186	.390
18	.828	1.477	.649
22	.655	1.489	.834
24	.852	1.606	.954
48	.799	1.603	.804
66	.745	3.032	2.282

TABLE A4 (a) Colony counts of revertants after treatment for different times with hycanthone at 0.125 mg/ml, IA-4 at 0.5 mg/ml, and buffer pH 5.9

Condition	Treatment time (hrs)	Colony Counts				locus
		YEPD	-his	-thr	-lys	
Buffer pH 5.9	0	1469	188	4	117	4
	8	1537	201	3	137	0
	16	1467	207	6	117	2
	25.5	1430	199	2	121	2
	35.5	1225	207	4	130	0
	46	1571	196	10	133	0
	60	1357	209	6	130	0
	71	1331	187	8	121	2
	112	1396	192	5	108	0
	161	1318	142	3	107	0
Hycanthone pH 5.9	0	1593	186	5	122	0
	8	1446	430	21	139	0
	16	1409	678	31	132	3
	25.5	1074	990	27	128	3
	35.5	825	1445	50	130	4
	46	622	1722	56	158	9
	60	552	1300	25	131	8
	71	320	811	18	95	7
	112	150	542	9	64	1
	161	83	200	7	35	1
IA-4 pH 5.9	0	839	120	1	18	0
	8	682	87	2	10	0
	16	807	124	2	10	0
	25.5	523	70	3	6	0
	35.5	490	43	3	9	1
	46	324	30	2	7	0
	60	312	40	2	2	0
	71	330	38	1	4	2
	112	226	32	0	6	0
	161	116	2	0	0	0

TABLE A7 Colony counts of revertants after treatment with hycanthone at 0.125 mg/ml, IA-4 at 0.5 mg/ml, buffer at pH 5.9, and after washing four times (Exp. I)

Condition	Treatment time (hrs)	Colony Counts			
		YEPD	-his	-thr	-lys
Buffer pH 5.9	0	1135	158	5	89
	12	1213	147	3	96
	24	1209	124	8	117
	48	1190	108	4	80
	66	1100	131	3	78
Hycanthone pH 5.9	0	1179	166	4	105
	12	1052	537	19	118
	24	1039	1366	55	160
	48	361	1226	34	91
	66	136	585	34	32
IA-4 pH 5.9	0	728	85	1	7
	12	397	42	3	8
	24	408	47	1	4
	48	268	28	0	2
	66	-225	64	32	16

TABLE A8 Colony counts of revertants after treatment with hycanthone at 0.125 mg/ml, IA-4 at 0.5 mg/ml and buffer pH 5.9 and after washing four times

Condition	Treatment time (hrs)	Colony Counts			
		YEPD	-his	-thr	-lys
Control 5.9	0	954	99	7	215
	9.5	923	80	4	247
	24	963	85	8	241
	33.5	961	81	4	257
	54	908	86	5	236
	73	954	88	4	257
Hycanthone pH 5.9	0	939	79	7	228
	9.5	961	206	12	253
	24	805	642	23	228
	33.5	616	1520	42	248
	54	311	933	49	135
	73	125	277	12	63
IA-4 pH 5.9	0	539	46	2	7
	9.5	365	27	2	2
	24	218	21	1	1
	33.5	210	29	0	1
	54	110	4	0	0
	73	87	11	0	0

TABLE A9 (a) Colony counts of revertants after treatment with various concentrations of hycanthone for eight hours

Hycanthone mg/ml	Colony Counts				
	YEPD	-his	-thr	-lys	locus
0	1206	124	2	80	1
0.031	1300	180	0	80	1
0.062	1086	276	8	113	3
0.125	1079	545	26	94	4
0.250	1127	826	37	113	2
0.50	784	891	46	91	4
1.00	522	586	17	27	2
2.00	261	144	9	10	1

TABLE A9 (b) Survival and reversion frequencies after treatment with various concentrations of hycanthone for eight hours

Hycanthone mg/ml	E.D.	Survival % of control	*his1-7* revertants ($\times 10^6$)	*hom3-10* revertants ($\times 10^7$)	Total *lys1-1* revertants ($\times 10^6$)	*lys1-1* locus revertants ($\times 10^8$)
0	0	100	1.03	0.17	0.66	0.83
0.031	0.248	100	1.39	0	.62	0.77
0.062	0.496	90.1	2.54	0.74	1.04	2.76
0.125	1.00	89.6	5.05	2.41	0.87	3.71
0.250	2.00	93.5	7.33	3.28	1.00	1.78
0.50	4.00	65.1	11.37	5.87	1.16	5.10
1.00	8.00	43.3	11.23	3.26	0.52	3.83
2.00	16.00	21.7	5.52	3.45	0.38	3.83

TABLE A10 (a) Colony counts of revertants after treatment with various concentrations of hycanthone at pH 7.0 for 12 hours

Hycanthone mg/ml	Colony Counts YEPD	-his	-thr	-lys	locus
0	1411	148	5	88	3
0.031	1388	217	6	106	0
0.062	1237	390	13	102	2
0.125	1157	841	40	142	5
0.250	996	1388	68	125	4
0.50	834	1564	84	96	6
1.00	595	1004	42	49	2
2.00	2.7	305	11	19	1

TABLE A10 (b) Survival and reversion frequencies after treatment with various concentrations of hycanthone for 12 hours

Hycanthone mg/ml	E.D.	Survival % of control	*hisI-7* revertants (x 10^6)	*hom3-I0* revertants (x 10^7)	Total *lysI-I* revertants (x 10^6)	*lysI-I* locus revertants (x 10^8)
0	0	100	1.05	0.35	0.62	2.13
0.031	0.372	98.4	1.56	0.43	0.92	0
0.062	0.744	87.7	3.15	1.05	0.83	1.62
0.125	1.500	82.0	7.27	3.46	1.23	4.32
0.250	3.00	70.6	13.94	6.83	1.26	4.02
0.50	6.00	59.1	18.75	10.07	1.15	7.19
1.00	12.00	42.2	6.87	7.06	0.82	3.36
2.00	24.00	15.4	4.06	5.07	0.88	4.61

TABLE A11 (a) Colony counts and survival and reversion frequencies, calculated after treatment with buffer, pH 7.0, under conditions of darkness

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1540	473	6	213
5.5	1404	449	6	186
9	1399	477	4	167
14.5	1393	486	5	201
17	1511	520	2	208
22	1472	531	3	233
34	1418	0	0	180

TABLE A11 (b)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	100	3.07	0.39	1.38
5.5	91.2	3.20	0.43	1.33
9	90.8	3.41	2.29	1.19
14.5	90.5	3.49	0.36	1.44
17	98.1	3.44	0.13	1.38
22	95.6	3.61	0.20	1.58
34	92.1	--	0	1.27

TABLE A11 (c) Colony counts, survival, and reversion frequencies after different hours of treatment with hycanthone under conditions of darkness

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1424	474	6	205
5.5	1196	700	21	212
9	1275	1004	49	153
14.5	1089	1164	63	176
17	986	1114	42	161
20	1057	1272	53	142
34	601	1380	77	95

TABLE A11 (d)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	92.5	3.33	0.42	1.44
5.5	77.7	5.85	1.76	1.77
9	82.8	7.88	3.84	1.70
14.5	70.7	10.69	5.79	1.62
17	64.0	11.30	4.26	1.63
20	68.6	12.03	5.01	1.34
34	39.0	29.62	12.81	1.58

TABLE A11 (e) Colony counts, survivals, and reversion frequencies after treatment with buffer, pH 7.0, in room lighting

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1354	549	5	202
5.5	1348	514	2	184
9	1412	492	4	192
14.5	1321	460	12	187
17	1369	524	11	217
22	1419	570	8	185
34	1318	500	5	186
54.5	1062	447	4	189

TABLE A11 (f)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	100	4.08	.372	1.50
5.5	100.2	3.81	.184	1.37
9	105	3.48	.283	1.36
14.5	98.2	3.48	.908	1.42
17	106.8	3.83	.804	1.59
22	105.5	4.02	.564	1.30
34	99.0	3.79	.379	1.41
54.5	79.0	4.21	.377	1.78

TABLE A11 (g) Colony counts, survival, and reversion frequencies after treatment with hycanthone for different hours, in room lighting

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1369	537	7	201
5.5	1226	889	37	203
9	988	1144	48	189
14.5	972	1432	64	132
17	712	1588	66	108
20	588	1580	77	109
34	192	1040	15	52
54.5	38	68	4	25

TABLE A11 (h)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	101.8	3.92	0.45	1.47
5.5	91.2	7.25	3.02	1.66
9	73.5	11.58	4.86	1.91
14.5	72.3	14.73	6.58	1.36
17	52.9	22.30	9.27	1.52
20	43.7	26.87	13.10	1.85
34	14.3	37.81	7.82	2.71
54.5	2.9	17.90	10.53	6.58

TABLE A11 (i) Colony counts, survival, and reversion frequencies after treatment with buffer pH 7.0 for different hours, in bright lighting

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1419	477	6	190
5.5	1367	486	9	203
9	1539	486	2	203
14.5	1230	501	3	190
17	1332	543	8	203
22	1228	482	4	200
34	1357	456	2	167
54.5	1267	516	5	176

TABLE A11 (j)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	100	3.36	0.42	1.34
5.5	96.3	3.56	0.66	1.49
9	108.5	3.16	0.13	1.32
14.5	86.7	4.07	0.24	1.55
17	93.9	4.07	0.60	1.52
22	86.5	3.93	0.33	1.63
34	95.6	3.36	0.15	1.23
54.5	89.3	4.07	0.40	1.39

TABLE A11 (k) Colony counts, survival, and reversion frequencies after different hours of treatment with hycanthone in bright lighting

Treatment time (hrs)	Colony Counts			
	YEPD	-his	-thr	-lys
0	1399	484	6	182
5.5	1197	849	45	163
9	1010	1282	65	166
14.5	578	1610	73	123
17	449	1588	82	114
22	431	1482	74	108
34	71	278	3	2
54.5	49	178	3	23

TABLE A11 (l)

Treatment time (hrs)	Survival % of control	Reversion Frequencies		
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)
0	98.6	3.46	0.43	1.30
5.5	84.4	7.09	3.76	1.36
9	71.2	12.69	6.44	1.64
14.5	40.7	27.86	12.63	2.13
17	32.6	35.37	18.26	2.54
22	30.4	34.39	17.17	2.51
34	5.0	39.16	42.25	.28
54.5	3.5	36.33	61.22	4.69

TABLE A12 (a & b) Colony counts, survival, and reversion frequencies after gamma-ray treatment

Dose (Krad)	Colony Counts				
	YEPD	-his	-thr	-lys	locus
0	12489	2014	68	859	5
4.1	6788	7972	268	1014	191
8.2	3372	6824	167	553	114
16.4	922	2899	117	289	58
24.6	411	1513	93	133	45
32.8	437	1762	93	160	56

Dose (Krad)	Survival % of control	Reversion Frequencies			
		his1-7 (x 10^6)	*hom3-10* (x 10^7)	*lys1-1* (x 10^6)	*lys1-1* locus (x 10^8)
0	100	1.61	0.54	0.69	1.20
4.1	54.4	11.74	3.95	1.49	28.13
8.2	27.0	20.24	4.95	1.64	33.81
16.4	7.4	31.44	12.69	3.14	62.91
24.6	3.3	36.81	22.63	3.24	109.49
32.8	3.5	40.32	21.28	3.66	128.15

TABLE A13 (a) Frequency of revertant cells from a requirement for arginine (Exp. I)

Concentration Hycanthone mg/ml	Number of Colonies on YEPD	Survival % of control	Number of Prototrophs on -Arg	Revertants ($\times 10^4$)
0	1212	100	208	1.72
0.031	1169	96.5	201	1.72
0.062	1127	93.0	215	1.91
0.125	1170	96.5	277	2.37
0.250	1035	85.4	390	3.77
0.50	1018	84.0	466	4.58
1.00	712	58.8	367	5.15
2.00	388	32.0	202	5.21

TABLE A13 (b) Frequency of red variant colonies among total colonies scored (Exp. I)

Concentration Hycanthone mg/ml	Number of Colonies on YEPD	Number of red sectors and colonies	Frequency of red variants ($\times 10^3$)
0	1212	5	4.13
0.031	1169	6	5.13
0.062	1127	5	4.44
0.125	1170	6	5.13
0.250	1035	13	12.56
0.50	1018	13	12.77
1.00	712	10	14.04
2.00	388	5	12.89

TABLE A13 (c) Frequency of revertant cells from a requirement for arginine (Exp. II)

Concentration Hycanthone mg/ml	Number of Colonies on YEPD	Survival % of control	Number of Prototrophs on -Arg	Revertants ($\times 10^4$)
0	1136	100	231	2.03
0.031	1134	100	208	1.83
0.062	1136	100	273	2.40
0.125	1217	107.1	319	2.62
0.250	1067	94.0	407	3.81
0.50	988	87.0	505	5.11
1.00	818	72.1	440	5.38
2.00	459	40.4	251	5.47

TABLE A13 (d) Frequency of red variant colonies among total colonies scored (Exp. II)

Concentration Hycanthone mg/ml	Number of Colonies on YEPD	Number of red sectors and colonies	Frequency of red variants ($\times 10^3$)
0	1136	1	0.88
0.031	1134	2	1.76
0.062	1136	4	3.52
0.125	1217	10	8.22
0.250	1067	8	7.50
0.50	988	11	11.13
1.00	818	13	15.89
2.00	459	9	19.61

TABLE A14 (a) Frequency of revertant cells from a requirement for arginine

Dose (Krad)	Colony Count YEPD	Survival % of control	Number of prototrophs on -Arg	Frequency	-control
0	4171	100	957	2.29	--
4.1	4154	99.6	1957	4.71	2.42
8.2	3819	91.6	2231	5.84	3.55
16.4	2861	68.6	2550	8.91	6.62
24.6	2207	52.9	2239	10.15	7.85
32.8	1651	39.6	2256	13.67	11.37

TABLE A14 (b) Frequency of red variant colonies among total colonies scored

Dose (Krad)	Colony Counts YEPD	Number of red variant colonies	Frequency of red variants ($\times 10^3$)
0	4171	18	4.32
4.1	4154	82	19.73
8.2	3819	100	26.18
16.4	2861	106	37.05
24.6	2207	117	53.01
32.8	1651	83	50.27